Testosterone Induction of Male Typical Song and Associated Neural Plasticity in Adult Female Canaries and European Starlings

By

Melvin L. Rouse, Jr.

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ABSTRACT

We have learned much from the songbird model. As the foremost animal model of vocal learning it has helped to elucidate the intricacies of neural control/coordination of musculature and respiration in vocal production. It has also clearly demonstrated the role of sensory perception and experimentation in vocal-motor sequence learning. The current set of experiments expands the impact of the songbird model by investigating these proximal mechanisms from the vantage point of sexual differentiation and adult neuroplasticity. The female songbird, in these experiments, demonstrates the sheer power of steroids in modifying behavioral output and the neural substrate of social/reproductive behaviors. In treating adult female songbirds of particular species with testosterone (T) one can study the effects of testosterone on sensorimotor learning: that being said, there are many remaining questions. There are three fundamental questions addressed in this thesis; 1) what is the trajectory of vocal development (i.e. sensorimotor learning) in T-induced adult female song and is it different from what occurs in T treated adult male song? 2) Does reproductive state limit the effectiveness of T to induce singing behavior in adult female songbirds? 3) What neural substrates are essential for the induction of song and the recapitulation of sensorimotor learning in adult females? Though male canaries showed had a shorter latency to respond to T-treatment by singing, female canaries did not differ from males in the overall pattern of song learning. However, T masculinizes female canary song only to a certain extent; sexually receptive reproductively active adult female canaries preferred to solicit in response to male T-treated song compared to female T-treated song. Reproductive state modulated the efficacy of T in inducing song behavior in adult female starlings. Lesions targeted to the

anterior forebrain pathway increased syllable stereotypy and blocked song learning in T-treated female canary. T-treatment modulated NR2A and NR2B immunoreactivity in the anterior forebrain in association with increases in singing. These findings suggest that though there are elements of song phenotype that cannot be masculinized or de-feminized in adulthood, steroids can activate pathways that are shared by males and females. This activation of latent pathways in females results in a different pattern of sexually differentiated brain and behavior, a pattern that in the future can be used to ask basic questions about the proximal control of learned song behavior.

ACKNOWLEDGMENTS

When I was around eight years old my dad gave me the nickname “Mr. Professor,” after several minor feats of technical mastery like reassembling and programing an early model VCR (never mind how it got disassembled…). I suppose inspired by these insights my mom required I visit the library weekly and write reports about the books I read. While there I would get lost in a world of “kid” science books about animals, the microbial world, astronomy and physics. I dreamed about science, about being one of the few people in the world who had the creativity and expertise to answer the unasked questions and share that knowledge freely with anyone who cared to listen. I have wanted to be a scientist for as long as I can remember.

There are no amount of words that can convey what this text means to me. The work that went in to this dissertation spanned years of my life; years that have had exhilarating highs and debilitating lows. While on this journey I have found love, started a family, lost cherished family members, questioned my abilities and intellect, and skirted the invisible line that separates actual modesty from timorousness. I am proud of this text. I am proud of what it represents.

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CHAPTER 1

THE CONTROL OF SONG IN PASSERINE SONGBIRDS

The song of birds is nature's music and has long served as a source of inspiration for artist and scientist alike. Many musical works of the classical era (1750-1830) of western art music featured motifs inspired by birdsong. Around the same time that this happened, in the late 1700s birdsong emerged as an area open for experimental manipulation (for reference see Barrington, 1773). One of the most intriguing facets of birdsong, particularly for modern behavioral neuroscientists, is that it is learned in a manner that is akin to human language (Doupe and Kuhl, 1999; Ball and Hulse, 1998). This aspect of birdsong was also recognized many years ago. Daines Barrington (1773) in a letter to the Phil. Trans. Royal Society of London presented some of the earliest evidence that vocal development in songbirds could be guided by learning. Barrington (1773) conducted a cross-fostering experiment and demonstrated that juvenile linnets (*Acanthis cannabina*) could learn the song of their skylark (*Alauda arvensis*) foster-parents.

Almost two centuries later, groundbreaking work from the likes of William Thorpe, Peter Marler, Mark Konishi, and others laid the foundation for our current understanding of how song is learned, its capacity limits, and what mediates singing in passerine songbirds (Marler, 2004, 2008). Furthermore, with the discovery of the song control system in the mid 1970's by Nottebohm and colleagues (1976) a neural substrate of the learning, production, and perception of this behavior has been identified. Birdsong

and the song control system have thus emerged as a valuable model for the study of brain-behavior relationships.

However, studies of song learning and development have primarily been focused on male songbirds as there are robust male biased sex-based differences (i.e. males > females) in the rate, quality, and neural substrate of singing; particularly, for temperate zone songbird species. However, there is great variation in the naturally occurring behavior of temperate zone female songbirds. Furthermore, there is also marked variation in the degree to which adult female songbirds can be pharmacologically (via sex-steroid action) pushed into male-typical patterns of brain and behavior.

In temperate zone songbird species singing often occurs in a seasonal context relating to cyclical changes in endogenous levels of circulating testosterone (see Ball, 1999; Tramontin and Brenowitz, 2000 for reviews). In addition, exogenous administration of testosterone can induce a cascade of genomic, neurochemical, and morphological changes related to many behaviors including song (Ball et al. 2002; Saldanha and Schlinger, 2008). It was observed in the mid-twentieth century that treating adult females with testosterone could induce male-like song (Leonard, 1939; Shoemaker, 1939). Nottebohm (1980) established that these effects of testosterone on song behavior were associated with changes in morphology of the song system in females in that the volumes of key forebrain nuclei became more male-like. These original findings in female canaries were surprising, in that it was not thought that steroid hormones could induce such dramatic changes in the brain and behavior of adults (Phoenix, et al, 1959). Rather, it was thought that the differentiation of a male-typical versus female-typical phenotype was based on the perinatal actions of steroids that produced the *organizational*

effects on brain and behavior (Phoenix, et al., 1959; Wade and Arnold, 1996; Saldanha, et al, 2000; Morris, et al, 2004). As compared to the number of studies on testosterone effects in males (e.g. Arnold, 1975; Wingfield, 1985; Nottebohm, et al, 1987; Marler, et al, 1987, 1988; Nowicki and Ball, 1989; Wiley, et al, 1993; Ball and Bernard, 1996; Bernard and Ball, 1997; Smith, et al, 1997; Gulledge and Deviche, 1998; Duffy, et al, 2000; Soma, et al, 2002; Sartor, et al, 2005) relatively little work has been done on testosterone effects in adult females. Most subsequent work investigating the effects of testosterone on adult female canaries has focused on the cellular consequences of these effects (e.g. Goldman and Nottebohm, 1983; Louissaint, et al, 2002; Hartog, et al, 2009). This dissertation will investigate the effects of testosterone (T) on vocal development in adult female songbirds, the neural substrate that underlies singing, as well as constraints on the *activational* properties of T.

In the remainder of this chapter, I will discuss the overall organization and functional characteristics of the song control system, with particular attention to key nuclei that comprise two discrete pathways in the songbird brain. Furthermore, I will discuss the implications of current research findings on the role of the anterior forebrain pathway of the song system in sensorimotor song learning. In addition, we will discuss the general effects of testosterone (T) on the brain and behavior of adult female temperate zone songbirds that sing in response to exogenous administration of T. Finally, in the experimental overview, a series of questions and experiments will be laid out the purpose of which will be to help disentangle the hormonal/neurochemical control of adult female patterns of sensorimotor vocal development and its neural substrate.

THE SONG CONTROL SYSTEM

The neuromuscular control of singing in songbirds is well defined and since the mid-1970s our understanding of the functional connections and utility of these nuclei have blossomed. Early tract tracing studies of the songbird brain illuminated in a step-wise fashion the motor afferents of the vocal organ of songbirds, the syrinx (Ball, et al, 2002; Nottebohm and Nottebohm, 1976; Nottebohm, et al, 1976). Subsequent studies formalized the functional links between the songbird brain and the neuromuscular control of singing. Generally speaking, the song system is composed of two pathways that are unique to songbirds, a vocal-motor pathway originating in the telencephalic nucleus HVC (used as its proper name) and an anterior forebrain pathway that is homologous to mammalian basal ganglia which also finds its anatomical origins in HVC (see Figure 1.1). The vocal-motor and anterior forebrain pathways control the learning, production, and maintenance of song in songbirds.

Of particular interest to psychology and neuroethology is the developmental process in which songbirds learn to sing, as it is strikingly similar to the way in which human language is acquired (Doupe and Kuhl, 1999; Jarvis, 2004). Prior to the discovery of the song control system classic experiments dating back to the 1960s, 1950s, and even the late 1700s demonstrated the remarkable ability of songbirds to learn their vocalization, a rare trait exhibited in a limited number of animal taxa (Barrington, 1773; Thorpe, 1954; Marler and Tamura, 1964; Konishi, 1965). Song develops in two primary phases; a sensory phase where nestlings hear the song of adults and an auditory memory is formed and a sensorimotor phase where the individual actively produces, evaluates, and alters its vocal output to match the vocal template formed in the sensory phase (Ball and Hulse, 1998). This basic understanding set the foundation for investigations of the

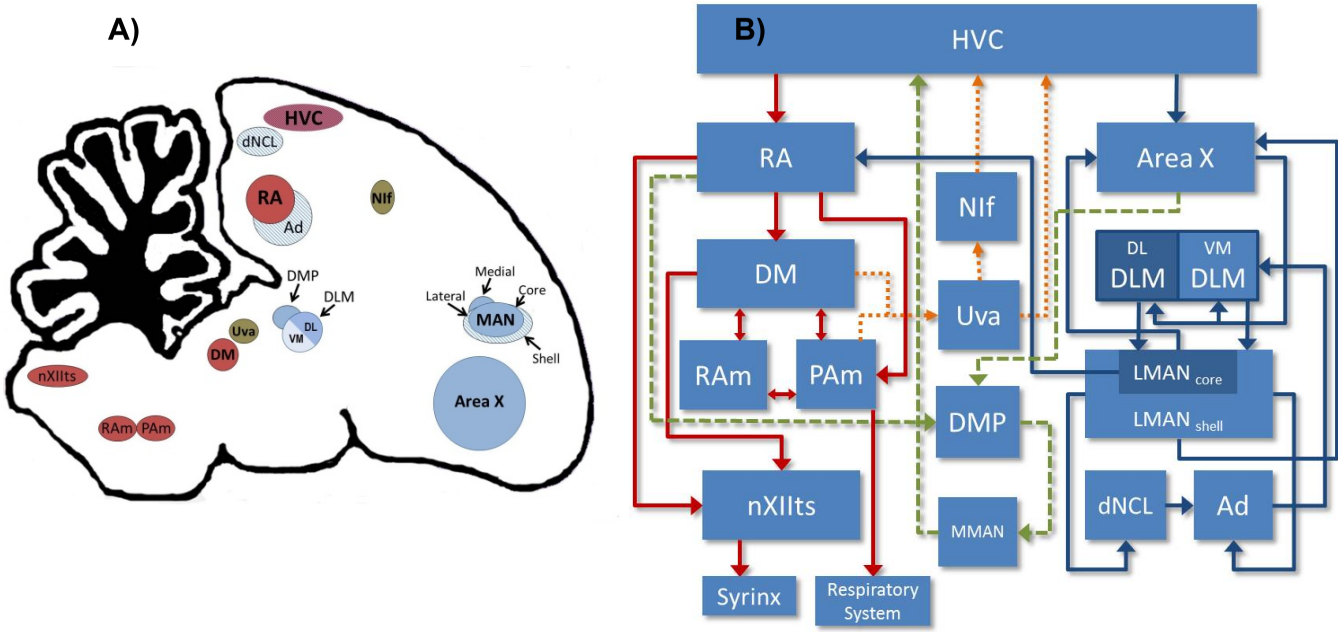


Figure 1.1: A) Singing in birds is controlled by a well-defined neural circuit, the song control system. It consists of nuclei found in the telencephalon, diencephalon, mesencephalon, and myelencephalon. **B**) The song control system is a recurrent network consisting of a caudal vocal-motor production pathway (red arrows), a bottom up feedback loop that sends an auditory and motor copy to HVC (used as its proper name; green arrows) as well as coordinate inter-hemispheric activity (orange arrows), and a rostral auditory forebrain feedback loop (blue arrows). HVC projects to RA which projects directly, and indirectly via DM, to nXIIts which in turn innervates the vocal organ of the songbird the syrinx. RA sends a motor copy to DMP which projects to MMAN which feeds back on to HVC. RA also projects to PAm which shares a reciprocal set of connections with RAm and DM. PAm and DM project to Uva which sends feedback projections directly, and indirectly via NIf, to HVC. HVC also projects to Area X which in turn projects to DLM. The dorsolateral (DL) portion of DLM projects to $LMAN_{core}$ while the ventromedial (VM) portion of DLM projects to $LMAN_{shell}$. $LMAN_{core}$ projects to RA. Both $LMAN_{core}$ and $LMAN_{shell}$ project to Area X (thereby forming a closed loop). Furthermore, $LMAN_{shell}$ projects to both dNCL and Ad. dNCL projects to Ad which in turn projects to the ventromedial portion of DLM forming another closed loop. **Abbreviations**: dorsal arcopallium (Ad), medial portion of the dorsolateral thalamus (DLM), dorsomedial nucleus of the intercollicular complex (DM), dorsomedial posterior nucleus of the thalamus (DMP), lateral magnocellular nucleus of the anterior nidopallium (LMAN), medial magnocellular nucleus of the anterior nidopallium (MMAN), nucleus interfacialis (NIf), the tracheosyringeal portion of the hypoglossal motor nucleus (nXIIts), nucleus paraambigualus (PAm), robust nucleus of the arcopallium (RA), nucleus retroambigualus (Ram), nucleus uvaformalis (Uva).

neural substrate of song in birds and furthermore highlighted the dynamic nature of brain behavior relations.

The following section will discuss in depth the neural control of song (starting with the vocal-motor pathway and ending with the anterior forebrain pathway), the developmental process of song learning, and hormonally induced singing in adult female songbirds. The review will then conclude with a justification for a set experiments the purpose of which are to push our current understanding of the interrelations of hormones, brain, and behavior in testosterone-induced singing in adult female songbirds.

THE VOCAL-MOTOR PATHWAY

The vocal motor pathway is directly involved in song production and contains the earliest discovered nuclei in the song control system. There is an overall top-down organization of this pathway. HVC projects to the robust nucleus of the arcopallium (RA) which then innervates structures in the midbrain and hindbrain (that are present in all avian species, including, the tracheosyringeal portion of the hypoglossal motor nucleus; nXIIts), which coordinate syringeal muscle movements with inspiration and expiration during singing.

HVC is among the earliest discovered structures in the song circuit. HVC is unique in that it is not only involved in the vocal-motor production of song but also in the learning and maintenance of this behavior. It is analogous to Broca's area in the human brain and complete lesions of HVC can yield aphasia-like symptomology where though the bird attempts to sing, it cannot; calls and other non-song vocalizations are not disrupted by HVC lesion (Vu, Mazurek, and Kuo, 1994). Nucleus HVC is located dorso-

medially in the caudal portion of the nidopallium. It is histologically characterized by dense clusters of neurons visualized by a number of staining techniques, including Nissl, tyrosine-hydroxylase, and met-enkephalin (ENK) immunoreactive staining. HVC is also characterized by high sex steroid receptor (both androgen and estrogen receptors) and aromatase expression (Ball, et al, 2002; Peterson, et al, 2005). HVC directly innervates RA; the RA projecting neurons are particularly sensitive to sex steroid action as these are the neurons incorporated in seasonally driven neurogenesis (Kirn, et al, 1991; Kirn and Nottebohm, 1993; Rasika, et al, 1994).

Nucleus RA was given its name because of the robust round clustering of large cell bodies observed in Nissl stained tissue (Nottebohm, et al, 1976). RA is located medially in the caudal extent of the arcopallium and is the most caudal song control nucleus in the telencephalon (Nottebohm, et al, 1976). The boundaries of RA as with HVC can be visualized by a number of techniques, including androgen receptor, vasoactive intestinal peptide (VIP), and ENK immunoreactive staining (Ball, et al, 1995).

RA is myotopically organized. Tract tracing studies of the neuromuscular control of the syrinx found that there is an orderly representation of nXIIts (Vicario and Nottebohm, 1988; Roberts, et al, 2007.). This general organization is reflected in projections from RA that synapse onto nXIIts, however, the myotopic topography observed in RA and its motor efferents is not observed in the RA projecting efferents from HVC (Vicario, 1991). This finding is surprising because this nucleus has been found to drive song production; lesions to HVC yield aphasia-like results (though the bird attempts to it cannot sing, however, it can make other vocalizations, i.e. calls; Nottebohm, et al, 1976; Simpson and Vicario, 1990).

NEURAL CODING IN VOCAL MOTOR CONTROL

HVC drives the production of song but sends an ultra-sparse code via its efferents (Hahnloser, et al, 2002; Fee, et al, 2004). During singing RA must send a continuous code to neuromuscular regions in the hindbrain for real time modulation of respiration and muscle movements (Hahnloser, et al, 2002). The premotor input RA receives from HVC must be translated via a not yet completely understood mechanism such that a sparse code is transformed into a continuous motor signal. The precise motor patterns generated by this continuous signal are what shape the acoustic units of birdsong through coordinated activation of many muscle groups (Suthers and Margoliash, 2002). Direct stimulation of HVC and RA in anesthetized birds results in the production of complex song-like vocalizations with species typical elements; however, there is no functional distinction between the two nuclei in this paradigm (Vicario and Simpson, 1995).

However, Vu and colleagues (1994) demonstrated that in awake-singing male zebra finch direct stimulation of RA alters syllable morphology though temporal characteristics of song remain the same. In contrast, stimulation of HVC in awake-singing male zebra finch disrupts only the temporal patterning of song; the syllable morphology is not altered (Vu, et al, 1994). Chi and Margoliash (2001) supported this finding by demonstrating that when male zebra finches direct their song toward a female the sub-millisecond spike burst timing in RA relates to the acoustic features of the song. These findings suggest that the coordination of muscle movement and breath which shape the acoustic features of song are under the control of RA, whereas, temporal patterns and the initiation of singing are under control of HVC.

BOTTOM-UP PROCESSING IN VOCAL MOTOR CONTROL

As previously stated, RA innervates structures in the avain midbrain (dorsomedial nucleus of the intercollicular complex [DM]) and hindbrain (nXIIts, nucleus retroambigualus [RAm], and nucleus paraambigualus [PAm]) which coordinate syringeal muscle movements with respiration (Ashmore, et al, 2008a,b; Wild, et al, 2000). When we look at this motor pathway as a whole we see that RA is a key nuclei as it is the interface where premotor signals are translated into varied combinatorial permutations of muscle movement and breath control. Furthermore, component parts of song behavior have lateralized control to a single hemisphere (Nottebohm and Nottebohm, 1976; Suthers and Margoliash, 2002). This lateralization of the control of component parts of a given behavior is common in vertebrate species (e.g. language in humans; Foundas, 2001; Geschwind, 1970). However, for complex behaviors like language or song because specific feature components are lateralized, activity between hemispheres must be coordinated for proper functional outcomes (Wild, et al, 2000).

Though it is known that different auditory processing characteristics are lateralized in the left and right forebrain of songbirds, all birds lack a large fissure like the mammalian corpus callosum. Generally speaking the left and right hemispheres of songbirds function independent of each other. However, inter-hemispheric coordination is needed for proper song production and recent data suggests that bottom-up processing relays/coordinates left/right activity. In particular, the vocal-respiratory network (VRN) is currently thought to be the source of this bottom-up processing as its unique circuitry,

pattern of activity, and placement within the vocal motor pathway strongly suggest it is responsible for inter-hemispheric coordination (Schmidt, 2008).

Data from the Schmidt lab suggest that VRN is the juncture point of bottom-up activation and coordination of bilateral activity in the control of song production (Ashmore, et al, 2008a,b). Three of the nuclei that RA innervates form VRN: DM, RAm, and PAm. Within the VRN there is a recurrent set of projections; RAm, PAm, and DM each have reciprocal connections with one another. DM is bilaterally active (Ashmore, et al, 2008a,b). VRN (via DM) sends a bottom-up signal ipsilaterally and contralaterally to the thalamic nucleus uvaformis (Uva), which innervates HVC directly and indirectly via nucleus interfacilis (Nif) (Ashmore, et al, 2008a,b). Furthermore, RA sends a projection to the dorsomedial posterior nucleus of the thalamus (DMP), which then projects ipsilaterally and contralaterally to the medial portion of the magnocellular nucleus of the anterior nidopallium (MMAN) which then projects to HVC (Ashmore, et al, 2008a,b).

Input from this bottom-up network to HVC is believed to set the temporal spacing for left and right control of the syrinx and respiration in the vocal-motor pathway. Bottom-up processing is poised to be the key in understanding how songbirds coordinate activity between hemispheres despite not having a grand fissure such as the mammalian corpus callosum. In sum, HVC receives left/right pre-motor signals from RA via MMAN while also receiving information on left/right motor activity from VRN. HVC on either hemisphere is thus able to monitor and coordinate patterns of activity without directly communicating with the other.

THE ANTERIOR FOREBRAIN PATHWAY

As with the vocal motor pathway the anterior forebrain pathway (AFP) originates in HVC. HVC projects to Area X which then projects to the medial portion of the dorsolateral thalamus (DLM; which for the purposes of this discussion we will only give a cursory notations), which projects to the magnocellular nucleus of the anterior nidopallium (MAN) which then innervates nucleus RA and feeds back onto Area X via projections from the lateral part of MAN (LMAN; Benton, et al, 1998; Foster and Bottjer, 1998). The Area X and LMAN circuitry form a recurrent loop and this circuit has a strong homology with mammalian basal ganglia. The anterior forebrain loop is involved with auditory processing and song learning.

Area X was given its name because at the time of its initial discovery it was not clear what functional role it played in song behavior. However, subsequent lesion studies demonstrated that Area X, as well as the other nuclei that form the AFP, though not critical for song production were essential for song learning and maintenance; this will be discussed in much greater detail later (Bottjer, et al, 1984; Scharff and Nottebohm, 1991; Sohrabji, et al, 1990).

Area X is located ventro-medially in the rostral portion of the telencephalon, the avian striatum. It was once a point of contention as to whether Area X was striatal or pallidal in composition; however, data from Allison Doupe and colleagues have made it abundantly clear that it is a chimera of sorts expressing characteristics of both (Carillo and Doupe, 2004). Area X shares a number of distinct similarities with mammalian striatum and pallidum; namely, there is cholinergic cell group expression, its projections are γ-aminobutyric acid (GABA)ergic, there is extensive dopaminergic innervation, as

well as direct projections to thalamic structures (Luo and Perkel, 1999a,b; Reiner, et al, 2004; Carillo and Doupe, 2004).

Area X is histologically characterized by the expression of neuropeptides commonly found in mammalian striatal spiny neurons, including substance-P and leu-ENK immunoreactive staining (Carillo and Doupe, 2004). It has a sparse but evenly distributed population of large glutamic acid decarboxylase (GAD) immunoreactive cells (Carillo and Doupe, 2004). In addition, there is expression of the protein product of the Nkx2.1 gene in Area X meaning that those immunopositive cells are distinctly pallidal in origin (Carillio and Doupe, 2004; Puelles, et al, 2000; Marin, Anderson, and Rubenstein, 2000; Rubenstein and Puelles, 1993). The unique composition of Area X strengthens the comparison of the AFP to mammalian basal ganglia as the mammalian circuit from cortex to thalamus traverses relay stations in both the striatum and the pallidum (Carillo and Doupe, 2004; Reiner, et al, 2004). In keeping with this homology between mammalian and avian basal ganglia the striato-pallidal complex Area X sends direct projections to the thalamus; more specifically the medial portion of the dorsolateral thalamus (DLM). DLM in turn innervates the lateral magnocellular nucleus of the anterior nidopallium (LMAN).

Anatomically, LMAN is located dorsally to Area X with its earliest sections becoming visible with rostral Area X. In addition, LMAN can be subdivided into two regions: $LMAN_{shell}$ and $LMAN_{core}$. LMAN is histologically characterized by a number of neurochemicals; however, the regional subdivision is often difficult to absolutely determine with a number neurochemical stains. Given this problem, boundaries for the complex is commonly collapsed across the overall visual border of LMAN proper.

LMAN can be visualized by staining for Nissl and NeuN-ir. In addition, the entirety of $LMAN_{core/shell}$ boundary can be clearly visualized with calbindin-ir staining (this is due to the axon terminals of DLM which richly express calbindin; Pinaud, Saldanha, Wynne, Lovell, and Mello, 2007). Furthermore, though both subdivisions express androgen receptors, only cells in $LMAN_{core}$ express met-Enk-ir and the calcitonin gene-related peptide-ir (CGRP-ir; Bottjer and Johnson, 1997; Bottjer, et al, 1997; Li, et al, 2006).

$LMAN_{core}$ projects to the motor nucleus RA (Bottjer and Johnson, 1997). $LMAN_{shell}$ projects to two nuclei the dorsal arcopallium (Ad) and the dorsal region of the caudolateral nidopallium (dNCL), we will return to this point later in the discussion (Reiner, et al, 2004; Bottjer and Altenau, 2010). However, both $LMAN_{core}$ and $LMAN_{shell}$ project to Area X forming a closed loop in the AFP (i.e. Area X, DLM, LMAN).

AFP FUNCTION IN BIRDSONG: AUDITORY FEEDBACK AND SONG LEARNING

The closed loop of Area X, DLM, and LMAN form the AFP which is the avian thalamocortical homologue of mammalian basal ganglia. The basal ganglia are a sight of sensorimotor integration and are critical for motor sequence learning. For a bird to learn to sing and to properly maintain song it must physically produce its vocalizations (i.e. motor) and then evaluate its own auditory output (i.e. sensory). The AFP is essential for the learning and maintenance of song.

Pre-dating the discovery of the song control system, in the mid-1960's song learning found its voice with Konishi's (1965) two-process model of song learning. In this model of song learning there are two essential phases, a sensory and a sensorimotor phase (Konishi 1965; Marler, 1990; Marler, 1997). During the sensory phase, nestlings hear the song of their parents or other adult conspecifics and an auditory memory or

'template' of song is formed (Marler, 1997; Peters, Marler, and Nowicki, 1992). Following the sensory phase is the sensorimotor phase. The sensorimotor phase of song learning can be thought of in three subdivisions: sub-song, plastic song, and crystallized song. Early on juvenile songbirds produce a sub-song that is soft in volume, bears little resemblance to adult vocalizations, and is quite distinct from feeding calls; it is akin to babbling in human infants (Marler and Peters, 1982). Young birds then produce a plastic song. This song is characterized by high phonologic variability and lacks the stereotypy of adult song (Marler and Peters, 1982). While engaging in plastic song, juvenile songbirds attempt to match their vocal output with the auditory memory formed during the sensory learning phase (Brainard and Doupe, 2002). These young birds will modify as well as drop off certain syllables that do not match the auditory template (Ball and Hulse, 1998; Brainard and Doupe, 2002) Auditory feedback is crucial particularly in the sensorimotor phase of song learning. Birds raised in isolation, though they learn to sing, tend to sing songs that are simpler and lack many species-typical elements (Brainard and Doupe, 2002). Furthermore, birds that are deafened during this phase of learning lack species-typical song elements, note stereotypy, and sing aberrant lengths of time (Konishi, 1965).

The AFP is specialized in the auditory processing necessary for learning and the development of complex species-typical song. Dan Margoliash (1983; 1992) discovered populations of cells within the anterior forebrain pathway specially tuned to fire only in response to the Bird's Own Songs (BOS). Consequent to this remarkable finding, studies investigated the function of these cells and anterior forebrain nuclei in mediating the song learning process.

Neurons in the AFP fire selectively to the birds own song as well as the tutor song in some cases (Solis and Doupe, 1999). Auditory neurons of the anterior forebrain of zebra finches become selective for song during song learning: as adults, these neurons respond more to the bird's own song (BOS) than to conspecific song or BOS played in reverse (Solis and Doupe, 1999). Interestingly, these neurons also fire in response to the tutor song (Solis and Doupe, 1999). This is not a surprising finding considering that the crystallized song of the adult zebra finch is based on the tutor meaning that there are many acoustic and structural similarities to the BOS. These similarities may be enough to elicit firing from these BOS tuned neurons. However, it was found that BOS tuned neurons of juvenile zebra finches that produced abnormal song following denervation of the syrinx fired both to the altered/abnormal BOS and the tutor song (Solis and Doupe, 1999). This finding suggests that BOS tuned neurons may fire to either signal or that that the abnormal song had not been produced long enough to completely retune these neurons. The latter is less likely particularly considering that over time only a portion of cells retuned to fire selectively to the abnormal BOS; other cells continued to fire to both signals despite acoustic dissimilarity.

BOS responses are present in HVC but not Field L, which is a homologue to the auditory cortex of mammals (Brainard and Doupe 2002). In LMAN, combination sensitive (i.e. BOS and tutor song) neurons have been found (Brainard and Doupe, 2002; Kao, Doupe, and Brainard 2005). Furthermore, data suggests that song selective neurons are highly non-linear and only respond to complex combinations in many cases meaning that the tuning of these neurons is set to the whole BOS and not constituent parts (Brainard and Doupe, 2002). Playing the entire initial sequence of BOS elicits a much

greater response than just playing individual syllables of BOS and adding their effect size (Brainard and Doupe, 2002). Taken all together, these data in combination with Konishi's two-process model of song learning led Allison Doupe to propose a functional model for the AFP, the error correction model.

SONG LEARNING: VOCAL VARIABILITY & LEARNING

As previously stated, in the sensorimotor phase of song learning juvenile birds are attempting to match their vocal output with the auditory memory they formed as nestlings during the sensory learning phase. To adjust vocal output while song is being produced, auditory nuclei must coordinate with motor nuclei sending an instructive*,* or rather, *error* signal to motor structures thereby altering the vocal output on a sub-millisecond scale. Auditory nuclei in this model act as a sensory evaluator conducting real-time monitoring of vocal output, comparing what the bird is singing to its template of song. This process is known as the error correction model and was proposed by Doupe and colleagues as a functional model for the AFP (Deregnaucourt, et al, 2004; Doupe, et al, 2005).

BOS tuned neurons in the forebrain are primed to play a key role in this model. It is these neurons and the combination sensitive neurons that are theoretically responsible for generating the *error* signal necessary to adjust vocal motor output. BOS tuned neurons in HVC and LMAN are particularly primed for a role in this model because both have direct projections to the motor control nucleus RA. Furthermore, LMAN, Area X and DLM constitute the closed feedback loop in the anterior forebrain. This point in particular makes LMAN a candidate nucleus to test the functional validity of this theory as HVCs' role in the temporal gating of song sequences has been demonstrated

previously (Hahnloser, et al, 2002). If specific neurons in HVC are tuned to BOS, or rather, fire increasingly to specific compositional features of BOS then neuronal clusters firing in HVC can temporally lock the muscular gestures of the syrinx and properly align syllable sequences that are sung such that BOS is reproduced reliably.

It was noted by Konishi (1965) that auditory input was necessary for proper vocal motor learning, in particular, deafening a bird prior to song crystallization yielded drastic abnormalities in song. Similarly, lesions to the anterior forebrain yielded abnormal song (Benton, et al, 1998). Interestingly, Brainard and Doupe (2000b) found that interruption of this pathway blocked the effects of deafening in adult songbirds. According to the error correction model, deafening would increase the error signal to motor pathways because though the bird is singing it can no longer hear and assess its vocal output. However, if the forebrain pathway is lesioned (assuming it is the generator of the error signal), the feedback necessary to propagate the error signal is cutoff; the forebrain loop is a closed circuit so a cut at any part blocks the processing of auditory input, or the lack thereof. Consequently, they found that lesions to the anterior forebrain pathway spare the effects of deafening on adult song.

Anthony Leonardo (2004), Bence Ölveczky and colleagues in the Fee lab (2005) formally tested the error correction model and found results that contradict that interpretation by Doupe and colleagues. As stated previously, LMAN is primed to play a potential role in the error correction model; it has BOS and combination tuned neurons, it is a part of the closed feedback loop in the forebrain, and it has a direct projection to the motor nucleus RA. Zebra finches were outfitted with specialized headphones such that when they sang, their vocal output was digitally altered and the auditory information they

received while singing did not match the motor output (Leonardo, 2004). If the error correction model were true it was theorized that spike patterns in BOS tuned LMAN neurons would shift firing thus instantiating an error signal to motor nuclei to adjust vocal-motor output. If the model was incorrect it was theorized that no such shift would be observed. BOS tuned neurons in LMAN did not respond to changes in auditory feedback, that is to say the pattern of firing remained constant irrespective of the auditory information received (Leonardo, 2004).

Similarly, data from Frank Johnson's lab found a corresponding result. Microlesions to HVC initially destabilized the song of adult male zebra finches (Thompson and Johnson, 2007). However, it was found that if LMAN were lesioned prior to HVC microlesions song remained stable (Thompson and Johnson, 2007). Together these data demonstrated that BOS firing in LMAN does not necessarily propagate an error signal. It was thus theorized that rather than an *error* (i.e. instructive) signal, LMAN propagates a jitter signal that induces variability in vocal-motor output (Thompson and Johnson, 2007; Olsvecky, Andalman, and Fee, 2005).

During the sensorimotor phase of song learning juvenile song birds vocally experiment. It is in this experimentation that they refine and hone their singing. As mentioned earlier, plastic song is characterized by high phonologic variability and lacks the stereotypy of adult song (Marler and Peters, 1982). During this phase young birds modify as well as drop off certain syllables that do not match the auditory template (Ball and Hulse, 1998; Brainard and Doupe, 2002). It was found in juvenile zebra finches that the source of this vocal experimentation is LMAN (Olsvecky, Andalman, and Fee, 2005). Prior to song crystallization if LMAN is selectively inactivated (in this case by sodium

ion channel blocker TTX) song becomes highly stereotyped and lacks phonologic variability a primary indicator of vocal experimentation (Olsvecky, Andalman, and Fee, 2005). This evidence also indicates that though HVC drives song behavior in general, LMAN contributes a jitter signal that introduces variability to vocal motor output. The variability that LMAN introduces to vocal output is necessary for song development; without this signal, motor sequences cannot be fine-tuned to exclude iterations that result in erroneous syllable production. In a way, LMAN aids in song development by driving the bird to make mistakes during learning.

Though there are several lines of evidence describing the role of LMAN in song learning these studies are somewhat limited in scope. The vast majority of these studies were done in juveniles strictly fixing interpretation in a developmental context. There are a number of species of songbird where the vocal repertoire grows and changes as the animal ages; with each season adult male canaries drop and add new syllables to their repertoire, likewise, as male European starling age their syllable repertoire grows (Nottebohm, Nottebohm, and Crane, 1986; Eens, 1997). Song develops at the same time as maturation of the reproductive system. LMAN is a target of steroid activity; it richly expresses androgen receptors. Testosterone (T) is required for the crystallization of song, the end product sensorimotor song learning (Marler, et al, 1988). Furthermore, though there are marked sex-based differences in the song behavior of many temperate zone species there is great variability in the ability of T to remodel the adult female system such that her brain and behavior (i.e. song) is masculinized. The current data does not sufficiently indicate LMANs role in adult patterns of sensorimotor learning. Additionally, the current data does not take into account the role of hormones in song learning (e.g. the

closing of sensitive periods and seasonal song crystallization, sex differences in song production, etc.).

SONG LEARNING: T AND THE FEMALE SONGBIRD

In some species of songbird, T-treatment in adult females can recapitulate sensorimotor learning. The earliest evidence that T-treatment induces male-like singing in adult female songbirds was presented by Samuel Leonard (1939) in a study published in the proceedings of the society for experimental biology and medicine. In this experiment, adult female canaries were procured from a local breeder and were injected intramuscularly in the breast with testosterone-propionate every 3 or 4 days until singing occurred (Leonard, 1939). The effects, however, were not permanent; once the injections ceased the birds stopped singing (Leonard, 1939). In the following issue of the proceedings of the society for experimental biology and medicine, H. Hurst Shoemaker (1939) reproduced these findings and detailed the behavioral changes that occurred relative to an oil-treated control group. It was found that relative to controls, adult female T-treated canaries sang more, performed male-typical courtship behavior, and displayed peck-dominance behavior over oil-treated females (Shoemaker, 1939). Some decades later Nottebohm (1980) replicated these behavioral findings and presented the first evidence that the song control nuclei of T-treated females relative to controls enlarged in response to exogenous T administration.

However, in addition to increasing the rate of singing, there is strong evidence indicating that testosterone recapitulates sensorimotor learning in females (Gahr and Garcia-Segura, 1996). Hausberger and Black (1991) described the similarities of song in

T-treated and non-treated control adult male and female European starlings. They found that T-treated female starlings produced songs closer in structure to males; however, the songs lacked some species-specific elements (Hausberger and Black, 1991; Hausberger, et al, 1995). Furthermore, they found that T-treated females had a song repertoire only slightly smaller than that of males (Hausberger and Black, 1991). These data suggest that T-treated adult female starlings re-engaged a sensorimotor learning program that culminated in a stark masculinization of song (Haesburger and Black, 1991; Hausberger, et al, 1995).

T induced changes in behavior are accompanied by changes in the brain (Nottebohm, 1980; Gahr and Garcia-Segura, 1996). The data are clear. In treating adult female songbirds of particular species with T one can study the effects of testosterone on a process of sensorimotor learning: that being said, there are many remaining questions. The data covered in this thesis aims to directly address these areas of concern by asking three fundamental questions:

1) What is the trajectory of vocal development (i.e. sensorimotor learning) in T-induced adult female song and is it different from what occurs in T treated adult male song?
2) Does reproductive state limit the effectiveness of T to induce singing behavior in adult female songbirds?
3) What neural substrates are essential for the induction of song and the recapitulation of sensorimotor learning in adult females?

Below I will introduce a series of experiments using two model species (the canary (Serinus canaria) and the European starling (Sturnus vulgaris)) that are designed to address these three fundamental questions.

EXPERIMENTAL OVERVIEW

The exogenous administration of T in adult females songbirds that respond to treatment by singing is not a completely well understood phenomenon. The factors that mediate the induction of song and the re-instantiation of sensorimotor song learning in T-treated adult female songbirds have not been systematically investigated. There are three primary questions that need be addressed to set the stage for future work to disentangle the neuro-modulatory factors that mediate individual aspects of song induction, learning, song circuit morphology alterations, and cell-molecular changes that take place in response to T in these birds.

Question 1: What is the trajectory of vocal development and does it differ from what occurs in T treated adult male song?

The first studies investigating the behavioral effects of T-treatment in adult female songbirds were done in the late 1930's and early 40's. Since then, we have gained a wealth of knowledge with regard to particular aspects of the activational properties of T in the adult female songbird brain. The field has since filled some of the gaps in our understanding of what, how, when, and where T is acting in the adult female songbird to enact these changes. However, some fundamental questions remain as to the time-course of behavioral development in females, what acoustic aspects of song are changing, the efficacy of T-induced song, and how this compares to T-effects in adult males?

Moreover, studies into the activational effects of T in adult female songbirds, for the most part, have focused on individual snapshots in time of behavior and do not overview the totality of the change that is induced in response to T over time. Investigations into the induction of song in adult T-treated female songbirds have not been systematic in this regard.

Fundamental to previous investigations of T-induced change in adult female songbirds is the assumption that males and females will respond in the same way to T-treatment and will undergo a process of development that is highly similar if not the same. However, these assumptions have not been empirically investigated and potential sex-based differences in the behavioral development and efficacy of T-treatment in adult male and female songbirds is wide-open for investigation. From this starting point we designed a set of experiments to formally and systematically ask, when given the same experimental manipulation(s) at the same time, do males and females react in the same way to T-treatment? In addition, is T-treated adult female song as efficacious a behavioral stimulus as T-treated adult male song?

These questions will be addressed in experiments 1 and 2. Experiment 1, discussed in chapter 2, is entitled, "Effects of Testosterone on Vocal Development and Neural Plasticity in Adult Male and Female Canaries (*Serinus canaria*): Sex differences in song induction and measures of song quality." Experiment 2, discussed in chapter 3, is entitled, "Is testosterone-treated adult female canary (*Serinus canaria*) song an effective behavioral stimulus?"

Question 2) Does reproductive state limit the effectiveness of T to induce singing behavior in adult female songbirds?

Singing in many temperate-zone songbirds (members of the avian order Passeriformes) varies dramatically in relation to sex and season (Catchpole and Slater, 1995; Kroodsma, 2005). The hormone testosterone (T) is involved in modulating many of these examples of intraspecific variation in song (Schlinger and Brenowitz, 2002). For example, song rate and quality is correlated with breeding condition in male songbirds (Brenowitz, 2004) and the application of exogenous T can dramatically increase singing and song complexity in many species (Harding, 2004). In the temperate zone, male songbirds commonly sing more complex songs at higher rates than females (Kroodsma, 2004, 2005; MacDougall-Shackleton and Ball, 1999). For example, in European starlings though both sexes can be heard singing in the non-breeding season, when day lengths are generally short; conversely though, only males typically sing during the breeding season when day lengths are long (Pavlova et al, 2005; Pavlova et al, 2007b). The stimulus control of song also varies between the sexes in starlings, the presence of a female increases singing rates in males; however, the presence of a male inhibits song in female starlings (Pavlova et al, 2007b; Riters, et al, 2000).

Interestingly in starlings as well as in some other songbird species such as canaries, treating adult females with T results in high rates of male-like singing (Shoemaker, 1939; Pesch and Guttinger, 1985; de Ridder, et al, 2002). However, one question that has yet to be asked is if this modulation of behavior is dependent upon particular endogenous factors such as reproductive state. Songbirds in the temperate zone exhibit remarkable seasonal plasticity in hormonal milieu, brain morphology, behavior, etc. Reproductive state changes seasonally and these endogenous factors differ depending on the state. T may only be effective in inducing a masculinization of adult female brain

and behavior if they are in a particular endogenous state. European starlings are highly sensitive to photoperiod and are more reliably and consistently pushed into particular reproductive states in the laboratory via photoperiod manipulations compared to other commonly studied species like domestic canaries. This second question will be addressed in experiment 3, discussed in chapter 4, the chapter of which is entitled, "Reproductive state modulates the effectiveness of testosterone to induce song behavior in adult female European starlings, *Sturnus vulgaris*."

Question 3) What neural substrates are essential for the induction of song and the recapitulation of sensorimotor learning in adult females?

LMAN is a key target of steroid activity; it richly expresses androgen receptors. LMAN plays a key role in song development. LMAN introduces a *jitter* signal into the motor program. This introduced variability facilitates the refinement of the motor sequence by shaping fixed patterns of activity in the motor afferents such that individual syllables become more stereotyped between and within bouts of singing. Increases in T are related to the induction of song learning and is required for the crystallization of song. T is thought to act in LMAN during song development to drive the aforementioned process. It is not known if LMAN plays a similar role in T-treated female songbirds that sing in response to treatment. Experiment 4 addresses this question and is discussed in chapter 5, which is entitled, "Lesions targeted to the anterior forebrain disrupt vocal variability in testosterone induced sensorimotor song learning in adult female canaries, *Serinus canaria.*"

Likewise, as previously stated, a well-defined neural circuit regulates singing in birds. Maturation of this circuit and the behavior, which it controls, develops at the same

time as maturation of the reproductive system. It has been shown in juvenile male zebra finch that there are age-dependent changes in the expression of N-methyl-D-aspartate receptors (NMDAR) in the anterior forebrain pathway (in particular, the subunits NR2A and NR2B; Basham, et al., 1999; Heinrich, et al., 2002, 2003). NMDARs are activated by the neurotransmitter glutamate, an excitatory neurotransmitter that is present in all vertebrates. These changes in NMDAR expression are thought to be essential for proper song development (Basham, et al., 1996; Wang and Hessler, 2006). Furthermore, it has been shown in adult male canaries that NMDAR subunit expression is seasonally regulated, thus, it is also related to fluctuations of circulating T (T-concentration is regulated seasonally as well; Singh, et al., 2003). It is not clear if this modulation in NMDAR expression occurs in adult female songbirds that sing in response to T-treatment or if it is related to T-induced singing in females. This question, experiment 5, will be addressed in chapter 6 and is entitled, "Testosterone modulates the expression of N-Methyl-D-Aspartate receptor (NMDAR) subtypes NMDAR-2A and NMDAR-2B in the forebrain adult female canaries, *Serinus canaria*."

The final chapter, chapter 7, will summarize the data and give concluding remarks as to female songbirds as a model of neuroendocrine controlled vocal-motor learning. Experiments 1 through 5 will be discussed at length in the following text.

CHAPTER 2

THE EFFECTS OF TESTOSTERONE ON VOCAL DEVELOPMENT AND NEURAL PLASTICITY IN ADULT MALE AND FEMALE CANARIES (SERINUS CANARIA): SEX DIFFERENCES IN SONG INDUCTION AND MEASURES OF SONG QUALITY

In temperate zone songbird species such as the canary (*Serinus canaria*), photoperiodic changes along with a variety of supplementary cues regulate the timing of the breeding season and modulate the associated anatomical, physiological, and behavioral changes (Nottebohm, 1981; Leitner, et al, 2001; Hurley, et al., 2008). In the spring, gonadal volume, as well as blood plasma concentrations of testosterone, increases with the increase in day length. This change in physiological state is sufficient, although not necessary (Ball, 1999), to facilitate an increase in song behavior (Schlinger, 1997; Ball, et al., 2003; Harding, 2004). This increase in song behavior during the breeding season is thought to serve two functions: mate acquisition and territory defense (Baker, et al, 1981; Catchpole, 1980, 1982; Darwin, 1871; King and West, 1977; Krebs, 1977; Kroodsma, 1976; Marler, 1956).

In many songbird species, males and females differ in the rate and quality of song as well as in the morphology of the controlling neural substrate (Nottebohm and Arnold, 1976; Nottebohm, 1981; Arai, et al., 1989; Kirn, et al., 1989; Brenowitz, et al., 1991; Whitfield-Rucker and Cassone, 1996; Smith, et al., 1995; Ball, et al., 2008). Male temperate zone songbirds tend to sing songs that are longer in duration, acoustically more complex, and produced at higher rates compared to females (Nottebohm, et al., 1981; Nottebohm, et al., 1986; Leitner and Catchpole, 2002). In wild and domesticated

canaries, *Serinus canaria*, this sex difference in the production of song is also observed (Poulsen, 1959; Leitner, et al, 2001). However, there is wide variation in the naturally occurring behavior of female temperate zone songbirds and in the ability of exogenous administration of testosterone (T) to pharmacologically induce in females male-like patterns of brain and behavior (Riebel, 2003; Harding, 2004). Adult female canaries, (that normally sing less frequent and simpler songs than males) for example, sing with greater frequency and quality in response to T-treatment (Gahr and Garcia-Segura, 1996; Hartog, et al, 2009). Likewise, the volumes of song control nuclei in T-treated adult female canaries tend to be larger than those of non-treated adult females (Nottebohm, 1980). T-treatment in adult female songbirds induces a cascade of molecular, morphological, and behavioral changes that appear to result in the recapitulation of the sensorimotor phase of song learning so that the ensuing song sounds more male-like (Nottebohm, et al., 1986, 1987). The activation of this seasonal process, which recapitulates a developmental learning stage typically observed in males, is supported by a masculinization of the brain of T-treated females.

In captive male canaries, naturally occurring changes in T concentrations are related to the development of song through the various stages of song development: subsong, plastic song and crystallized song (Nottebohm, et al., 1986; 1987). These changes in song behavior are causally related to T as T-treatment in adult male gonadectomized songbirds induces a similar cascade of molecular, morphological, and behavioral changes. T-treated castrates show increases in singing behavior that are similar to changes that are observed in intact photostimulated males. Moreover, in gonadally intact adult male canaries song repertoire changes and can increase across

seasons with the addition and deletion of syllable types as a function of age. These changes parallel and are partially caused by seasonal changes in T concentration (Nottebohm, et al, 1978, 1986; Leitner, et al. 2001).

T thus appears to have powerful effects on song behavior and the underlying brain regions mediating song behavior in male and female canaries. However, it is not known if male and female songbirds respond to T in the same way. When adult sex differences are mediated by differences in circulating steroid concentration (i.e. activational effects rather than organizational effects early in ontogeny), it is often assumed that males and females have similar capacities to respond to T. This is not necessarily the case. It is not known if the pattern of vocal development and associated changes in neural morphology is the same in T-treated male and female songbirds. This study systematically investigates the activational effects of varying doses of T-treatment in adult male and female canaries housed under the same photoperiodic conditions.

METHODS

Experimental animals

Forty-three male and forty-three female American singer canaries were obtained from a local breeder (Maryland Exotic Birds) and housed in an indoor aviary on an 8L:16D (light:dark) light cycle for at least eight weeks. Birds were kept in 49 x 95 x 51 cm cages (six birds per cage) at Johns Hopkins University, Baltimore, MD and fed canary food and provided water *ad libitum*. Care and handling of all animal subjects was in accordance with guidelines published by the National Research Council (2011) and all

experimental procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. After eight weeks on 8L:16D, females ovaries were regressed as confirmed by laparotomy and males with regressed testes were castrated under general anesthesia using isoflurane (mix of air gas and 3% isoflurane for induction and 2.5% during the surgery). The two testes were removed through a small incision in the flank, posterior to the last rib. The incisions were sutured closed and lidocaine and antibiotic ointment was placed on the wound. Males were allowed to recover under warm light before being returned to their home cages.

Males and females remained on an 8L:16D light cycle for at least eight additional weeks before they were implanted subcutaneously with one Silastic™ implant (Dow Corning, Midland, MI, USA, no. 602-175; 0.76 mm inner diameter, 1.65 mm outer diameter) of either 2 mm, 6 mm, 12 mm length filled with crystalline T or a 12 mm implant left empty as a negative control. Implant length and diameter were selected based on previously published studies in canaries indicating that these implant sizes are sufficient to activate singing and to increase song control nuclei size to levels characteristic of gonadally-intact males (Appeltants, et al., 2003; Nottebohm, 1980; Sartor, et al., 2005).

Starting immediately after these subcutaneous implantations, all male and female canaries were individually housed in sound attenuated chambers (41cm x 48cm x 51 cm) on an 8L:16D light cycle. Song behavior was recorded for thirty minutes, three times per day for either seven or twenty one days, depending on treatment group. Isolation chambers were outfitted with a microphone (BT-MP8087 Mini microphone; B&H Foto

and Electronics Corp, New York, NY) and camera (KPC-600 Pinhole Camera 3.6mm; B&H Foto and Electronics Corp, New York, NY) connected to computer running DVRserver (V6.33b; Mammoth Technologies, Austin, TX) designed for real-time video and audio surveillance recording. Behavioral recordings (audio and video) began immediately after lights on (0900 hrs), four hours after lights on (1300 hrs), and thirty minutes before light off (1630 hrs). All canaries remained on an 8L:16D photoperiod for the duration of the study as it was necessary to examine the effects of the steroid hormone treatment in the absence of any other cues characteristics of breeding condition.

Behavioral Quantification

Sound files (converted from audio/video file format .mp4 to audio only .wav files) were sampled at 22050 Hz, which translates to a frequency range of 0 to 11 kHz. Audio files were highpass filtered with audio editing software Goldwave ™ (version 5.55) set to a threshold of 900 Hz to remove low frequency noise (e.g. the sound of the fan/air vent and hum of the light). Sound spectrograms were created for each daily recoding using Avisoft SASlab (Avisoft Bioacoustics, Berlin, Germany). Spectrogram FFT (fast Fourier transform) lengths were set to 512 with an overlap of 75% for the temporal resolution. For each spectrogram the number of songs, song bout duration (in seconds), number of calls, the total time spent singing and/or producing vocalizations (i.e. calls in addition to songs), and other acoustic features (i.e. mean Wiener entropy, Wiener entropy variance, the number of elements per song bout, vocalization energy, and mean peak amplitude) for each recording were calculated and exported into an Excel spreadsheet.

Canary song has a characteristic acoustic structure and temporal pattern that distinguishes it from calls and we used this criterion to differentiate songs from calls in the spreadsheet (Guttinger, et al., 1978; Guttinger, 1984; Nottebohm and Nottebohm, 1976; Wolffgramm, 1973). The smallest discernable unit of song is the element (i.e. note); it is the smallest continuous structure in a spectrogram (Guttinger, et al., 1978). Multiple elements can be combined in a fixed pattern to form a syllable (Guttinger, et al., 1978; Wolffgramm, 1973). Elements and syllables are generally sung in fast rhythmical repetitions known as trills or phrases (sometimes also referred to as tours; Guttinger, et al., 1978; Nottebohm and Arnold, 1976). The temporal characteristics of song as well as acoustic features associated with song were such that we could reliably and accurately define song bouts automatically in the data set. We defined song as being bouts of vocalizations where the total duration was greater than 1.5 seconds of continuous elements (featuring 5 or more elements that have a peak amplitude value greater than -22dB) with inter-syllable intervals no longer than 500 milliseconds and a mean entropy value less than W = 0.550.

In addition to measures of song rate, other acoustic features of song were measured including the aforementioned Wiener entropy variance, the number of elements per song bout, vocalization energy, and mean peak amplitude. Energy is the sum (i.e. integral) of the squared amplitudes of a sound multiplied by its sampling time (Avisoft SASlab User Manual) and is a measure of the 'loudness' of a given vocalization. Unlike the peak amplitude the energy measure is less sensitive to the distance of the vocalization source to the microphone as it is collapsed across the total waveform rather than a single point measurement. Wiener entropy is a measure of the spectral width and uniformity of

a signal. It is a pure number (i.e. unitless) with 0 being a pure tone (e.g., a uniform sinusoidal wave) and 1 being noise (i.e. a non-uniform random signal; Tchernichovski, et al., 2000). In canaries we have found that the variance of this measure collapsed across a single bout of singing provides a good measure of syllable diversity and syllable stereotypy; high entropy variance tends to indicate songs with a high degree of vocal variability as well the inclusion of multiple syllable/phrase types (Unpublished observations). This is similar to what is observed in juvenile male zebra finch in which entropy variance increases rapidly with the onset of sensorimotor song learning, which is marked by significant vocal variability (both in note composition and spectral stability; Tchernichovski, et al., 2001; Shank and Margoliash, 2009).

In communication, the sender(s) of signals must broadcast in a range of social contexts and environmental conditions for which the receiver may or may not be within range for confirmation of signal reception (Todt and Naguib, 2000). It is often advantageous for the signaler in terms of fitness to increase the likelihood of the signal being received by increasing the amplitude or gain of the signal (Searcy and Nowicki, 2005). In the case of vocal communication, loud signals exploit the sensory modality and increase the likelihood of conspecific vocalizations being discriminated from background (Todt and Naguib, 2000; Searcy and Nowicki, 2005). Song in male songbirds functions both to attract a mate and defend a territory; males can modulate the amplitude of signals to maximize discrimination from background noise or to compete with other conspecifics (Brumm and Todt, 2002, 2004). To assess further potential sex differences in T-induction of song behavior and its relation to this aspect of song and vocal signaling, we statistically investigated whether males and females produced loud signals and complex

signals in a similar manner. We did this by correlating vocalization loudness (i.e. vocalization energy) and the rate/composition of song in both sexes.

Finally, we tracked the development of individual syllables to assess the sensorimotor learning process that occurred as the song over time became closer to a state of crystallization. When song crystallizes, individual syllable iterations of a given type within a bout of singing and between bouts of singing become highly similar to one another (Waser and Marler, 1977; Marler and Peters, 1982). Crystallized song is characterized by high stereotypy (Marler, 1997). To measure the rate of syllable development and crystallization we isolated high amplitude vocalizations (peak amplitude > -14dB; song elements only, calls omitted) on the final day of recording, day 21, into individual .wav files. Syllables were then randomly re-sampled (maximum 100 syllables), categorized, and template sonograms were made for selected syllable iteration. High amplitude vocalizations (peak amplitude > -14dB; song elements only, calls omitted) on the days 5, 10, 15, & 20 were then isolated into individual .wav files. Binary sonogram templates from the final day were then cross-correlated with sonograms for all song elements above amplitude threshold for days 5, 10, 15, & 20 in Avisoft SASlab. The percentage of syllable correlations above an r = 0.95 correlation threshold was then tabulated for each bird for each day (i.e. days 5, 10, 15, 20).

Serum T enzyme linked immunoassay (EIA)

Blood samples were collected into microcentrifuge tubes at the end of the experiment (after seven or twenty one days of treatment with T) from the severed carotid artery of the bird immediately after rapid decapitation. Blood was centrifuged at 9,000

RPM for 5 minutes resulting in separation of serum from red blood cells. Serum concentrations of T were measured using an enzyme linked immunoassay from Enzo Life Sciences (Testosterone EIA kit; cat #ADI-901-065, Plymouth Meeting, PA). The kit was validated for use with canary serum by testing for parallelism and recovery of added mass (standard biochemical validations). To test for parallelism, high and low T pools were pipetted at five different volumes in quadruplicate to ensure that the dose response curves were parallel to the standards under dilution and to confirm that T in the sample bound with the antibody with the same affinity as T in the standard curve and that no other compound in the sample binds to the antibody but with a different affinity. Recovery of exogenous testosterone verifies accurate measurement throughout the working range of the assay. To test for recovery of added mass, three standard curve points (from the middle of the curve) were added to the high and low pool to ensure that the added mass could be accurately detected, indicating that the sample was not blocking the antibodies ability to bind with the standard. The intra- and interassay coefficients of variation were 9% and 14%, respectively.

Song nuclei volume reconstruction

At the end of the treatment period (1 week or 3 weeks), birds were euthanized by rapid decapitation and brains were extracted and placed in fixative (5% acrolein). Brains were slightly agitated in acrolein fixative for two hours, rinsed (4 x 15 minutes) in 0.01M Phosphate Buffer-Saline (PBS; pH 7.4) and cryoprotected in 30% sucrose until saturated. Brains were then flash frozen on dry ice and placed in the -70°C until processed for later analysis.

Brains were sectioned at 30 μm thickness using a cyrostat (Carl Zeiss) and Nissl-stained using Thionin to visualize the song control nuclei HVC, Area X, and the robust nucleus of the arcopallium (RA). Brains sections were placed in 0.01 M PBS solution and mounted onto gelatin-coated microscope slides. The slides were dried, stained with thionin for five minutes, serially dehydrated in ethanol at 30%, 50%, 75%, 95%, 95%, 100% for one minute and a final step in 100% ethanol for five minutes. The slides were then cleared in xylene (Fisher Scientific) and coverslipped with Permount (Fisher Scientific).

Brain regions of interest were digitized using a bright field light microscope (Zeiss Axioscope, Carl Zeiss, Thornwood NY) with a CCD camera connected to a desktop computer. For each image, the area of the brain region was measured using Openlab 5.0.2 (Improvision, Lexington, MA). The volume of each region was then reconstructed combining the areas of subsequent sections with the sampling interval (120 μm) using the formula for a truncated cone (developed by Smith et al., 1995) as used previously in starlings (Bernard and Ball, 1995; Bentley et al., 1999; Bernard and Ball, 1997). For each bird we used the average volume of the nuclei measured in the left and right hemispheres.

Statistical Analysis

To evaluate the song rate, ratio of song to calls, and vocalization energy data we used a split-plot factorial analysis of variance (ANOVA; 4-way mixed-design with 3 between [i.e. sex, dose, & week of tissue collection] and 1 within subjects variable [i.e.

day of the week]). For these repeated measures variables, significance for main effects was corrected using a Greenhouse-Geisser correction for non-sphericity.

Daily mean values were highly variable and not normally distributed for song bout length and number of individual notes so marginal mean values for the entire week (i.e. week 1 and week 3) were used to decrease variance and normalize the data. For these two behavioral measures averaged for the entire week and for T concentrations in blood samples that were only collected at the end of the experiment, we thus only had one time point for analysis in each experimental group. Three-way analyses of variance were therefore used to analyze these data (i.e. sex, dose, & week of tissue collection). Three-way Bonferroni-corrected multivariate analysis of variance (MANOVA; independent variables included sex, dose, & week of tissue collection) was used to discern particular patterns in the data where there were multiple contiguous dependent variables such as the song control nuclei volumes (i.e. volume of HVC, Area X, and RA). All post-hoc tests were corrected for multiple comparisons using Bonferonni's correction unless otherwise noted.

Relationships (i.e. correlations) between variables were evaluated with linear regression analyses controlling for covariance of related predictors. Regressions analyses were run in the following order: males only during week 1, females only during week 1, males only during week 3, and females only during week 3. Values for the week of observation were means collapsed across the entire week. The reported values are the individual contribution of each variable to the final regression model(s). Results were considered statistically significant for $\alpha < 0.05$.

RESULTS

Testosterone concentration

We measured the post-treatment concentrations of T in circulating serum and found a significant of effect of dose ($F(3,72) = 4.885$, $p = 0.004$). However, there was no difference in serum T concentration between males and females nor between time of sampling (i.e. week 1 versus week 3; $F_{sex}(1,72) = 0.130$, $p = 0.720$; $F_{week}(1,72) = 0.724$, $p = 0.398$). Likewise, there were no significant interaction effects ($F_{sex*dose}(3,72) = 2.079$, $p = 0.113$; $F_{sex*week}(1,72) = 0.277$, $p = 0.601$; $F_{dose*week}(3,72) = 0.393$, $p = 0.758$; $F_{sex*dose*week}(3,72) = 1.376$, $p = 0.259$). Though the most powerful difference between doses was between the blank control versus the high dose ($i\text{-}j_{control\text{-}high} = -1134.959$, $p = 0.001$) there was a robust linear trend showing that the size of the implant was related to T concentration in serum ($p < 0.001$; See figure 2.1).

Song rate

Exogenous administration of T is known to increase the singing rate of castrated male and intact female canaries and we found a significant main effect of dose on this dependent variable ($F(3,68) = 3.438$, $p = 0.022$; see figure 2.2). Furthermore, there was a significant main effect of sex; males sang more than females ($F(1,68) = 8.792$, $p = 0.004$). Likewise, there was a significant main effect of week; birds sang more during week 3 than week 1 ($F(1,68) = 5.891$, $p = 0.018$). There were no significant interaction effects for the between subjects variables ($F_{sex*dose}(3,68) = 0.434$, $p = 0.729$; $F_{sex*week}(1,68) = 1.205$, $p = 0.276$; $F_{dose*week}(3,68) = 0.600$, $p = 0.617$; $F_{sex*dose*week}(3,68) = 0.511$, $p = 0.676$). Overall, birds treated with a low dose of T sang more frequently than

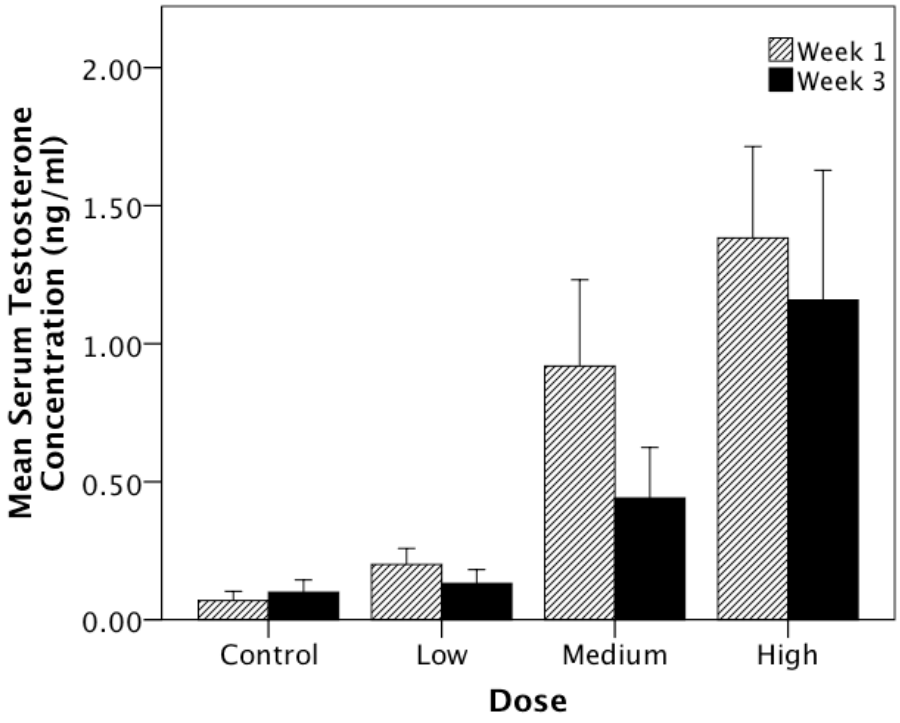


Figure 2.1: Effect of Silastic implants of various sizes filled with testosterone on serum testosterone concentrations in male and female canaries (data pooled) at one or three weeks after implantation. There was a significant effect of capsule size (i.e. dose) on serum concentrations of testosterone (T). T did not differ, however, by week of treatment or sex (i.e. males and females had equivalent levels of T in serum).

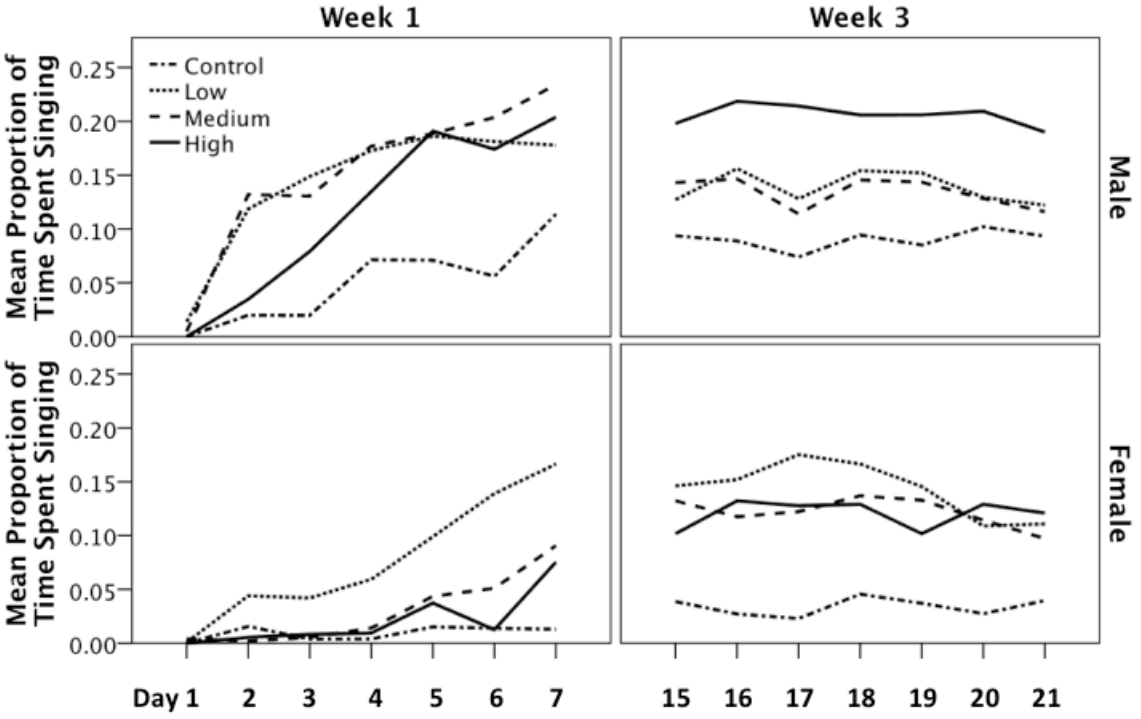


Figure 2.2: The effect of T-treatment on the rate of singing in males and females during week 1 and week 3. During week 1 of T treatment males exhibited shorter response latency to T-treatment (i.e. they sang sooner after T-treatment compared to females) and sang at a higher rate than females. However, by week three T-treated females sang at similar rates compared to males.

controls ($i\text{-}j_{control\text{-}low} = -0.080$, $p = 0.032$). There was a trend in the data for birds treated with a high dose of T to sing more often than controls, however, after Bonferroni correction for multiple comparisons the trend was not significant ($i\text{-}j_{control\text{-}high} = -0.070$, $p = 0.082$).

There was a significant main effect of the repeated measure, i.e. the day of observation ($F(6,408) = 23.980$, $p < 0.001$). As expected, however, there was a significant interaction between the day of observation and week of assessment ($F(6,408) = 31.807$, $p < 0.001$). There was also a significant interaction between the day of observation and sex ($F(6,408) = 5.761$, $p < 0.001$) and moreover, there was a significant three way interaction between sex, week of assessment, and day of observation ($F(6,408) = 5.589$, $p < 0.001$). All other interactions of the between and within subjects variables on singing rate were not significant ($F_{day*dose}(18,408) = 1.150$, $p = 0.327$; $F_{day*sex*dose}(18,408) = 0.924$, $p = 0.511$; $F_{day*sex*dose*week}(18,408) = 1.741$, $p = 0.073$).

Post-hoc analyses showed that though during week 1 there was a significant effect of day on singing rate ($F(6,246) = 31.728$, $p < 0.001$) during week 3 this effect was only a non-significant trend ($F(6,246) = 2.077$, $p = 0.089$; refer to figure 2.2). A Bonferonni corrected MANOVA for days 1 through 7 (i.e. week 1 of treatment) revealed that males sang more than females on days 2 through 7 of T-treatment ($F_{day1}(1,34) = 0.776$, $p = 0.385$; $F_{day2}(1,34) = 9.504$, $p = 0.004$; $F_{day3}(1,34) = 20.089$, $p < 0.001$; $F_{day4}(1,34) = 25.499$, $p < 0.001$; $F_{day5}(1,34) = 13.195$, $p < 0.001$; $F_{day6}(1,34) = 15.257$, $p < 0.001$; $F_{day7}(1,34) = 10.393$, $p = 0.003$). In contrast, a separate Bonferonni corrected MANOVA showed that males and females did not differ on any day of treatment in week 3 ($F_{day15}(1,34) = 0.878$, $p = 0.355$; $F_{day16}(1,34) = 1.535$, $p = 0.224$; $F_{day17}(1,34) = 0.340$, $p =$

0.564; $F_{day18}(1,34) = 0.605$, $p = 0.442$ $F_{day19}(1,34) = 1.378$, $p = 0.249$; $F_{day20}(1,34) = 1.925$, $p = 0.174$; $F_{day21}(1,34) = 1.340$, $p = 0.255$).

Ratio of songs versus calls

Over the recording period we measured and compared the rate of singing to the rate of calling. Analyses of this ratio revealed a significant main effect of dose ($F(3,68) = 5.532$, $p = 0.002$; see figure 2.3). Likewise there was a significant main effect of sex; males spent a greater proportion of time singing versus calling compared to females ($F(1,68) = 7.620$, $p = 0.007$). Furthermore, there was an effect of week; birds spent a greater proportion of time singing versus calling in week 3 compared to week 1 ($F(1,68) = 6.954$, $p = 0.010$). There were no significant interactions of the between subjects variables ($F_{sex*dose}(3,68) = 0.173$, $p = 0.914$; $F_{sex*week}(1,68) = 2.586$, $p = 0.112$; $F_{dose*week}(3,68) = 0.442$, $p = 0.724$; $F_{sex*dose*week}(3,68) = 0.204$, $p = 0.893$). At low and high doses T-treated birds spent a greater proportion of time singing versus calling compared to controls ($i\text{-}j_{control\text{-}low} = -0.361$, $p = 0.003$; $i\text{-}j_{control\text{-}high} = -0.331$, $p = 0.007$). There was also a trend for medium dose birds to spend a greater proportion of time singing versus calling, however, after a Bonferonni correction for multiple comparisons the finding was not statistically significant ($i\text{-}j_{control\text{-}medium} = 0.261$, $p = 0.061$).

There was a significant main effect of the day of observation on the ratio of song to calls ($F(6,408) = 29.787$, $p < 0.001$). However, there was a significant interaction between the week of assessment and the day of observation ($F(6,408) = 24.277$, $p < 0.001$). Likewise, there was a significant interaction between sex, week of assessment, and day of observation ($F(6,408) = 4.095$, $p = 0.003$). All other interactions of the

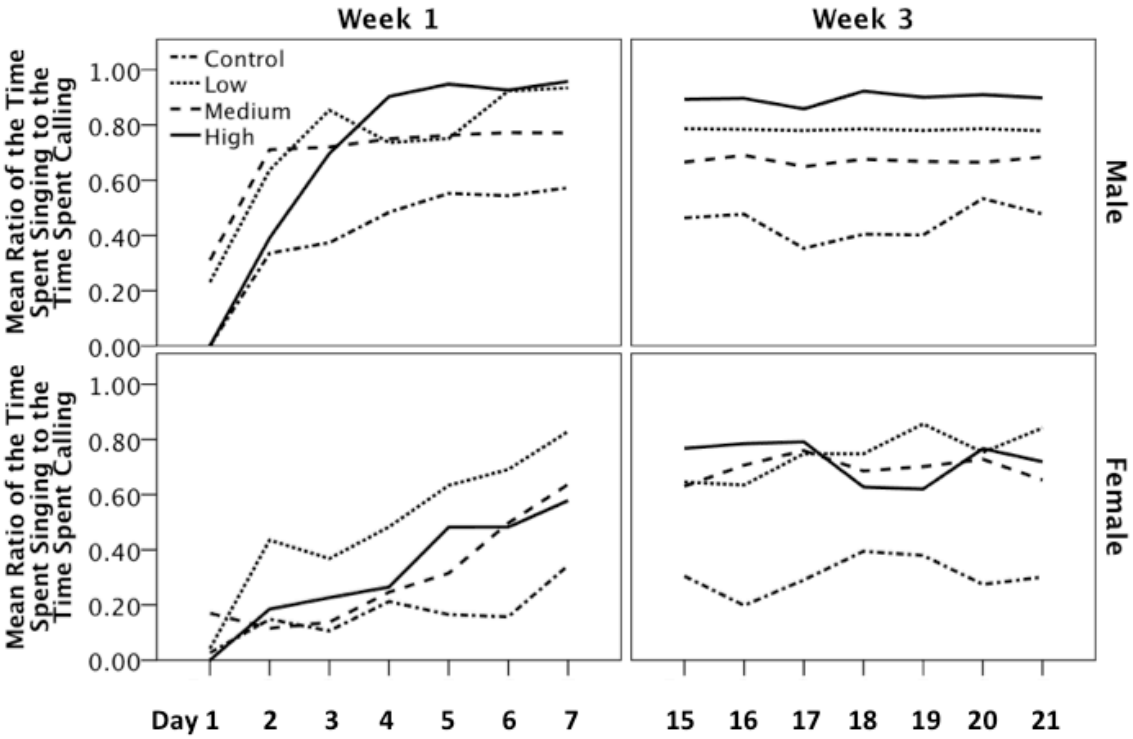


Figure 2.3: The effect of T-treatment on the ratio of time spent singing to the time spent calling in males and females during week 1 and week 3. T-treatment increased general vocalizing of all birds; however, during the first week females spent a lower proportion of that time singing versus calling compared to males. However, by week three the ratio of song to call was similar in males and females.

between and within subjects variables on the ratio of song to call were not significant ($F_{day*sex}(18,408) = 1.945$, $p = 0.104$; $F_{day*dose}(18,408) = 0.948$, $p = 0.499$; $F_{day*sex*dose}(18,408) = 1.513$, $p = 0.120$; $F_{day*dose*week}(18,408) = 1.544$, $p = 0.109$; $F_{day*sex*dose*week}(18,408) = 0.622$, $p = 0.821$). During week 1 of T-treatment females spent a greater proportion of time calling versus singing as compared to males but did not differ from males in the ratio of song to calls during week 3 (refer to figure 2.3; $F_{week1}(1,34) = 15.171$, $p < 0.001$; $F_{week3}(1,40) = 0.259$, $p = 0.613$). Furthermore, though there was a main effect of day of observation during week 1 of treatment ($F(6,204) = 38.122$, $p < 0.001$) the effect was not significant by week 3 ($F(6,204) = 0.328$, $p = 0.922$).

Mean song bout length

There was a significant main effect of dose on the mean song bout length (in seconds) averaged across all days of a given week (see figure 4; $F(3,67) = 6.208$, $p < 0.001$). In addition, there was a significant main effect of week; birds that sang during week 3 of observation sang longer songs than birds that sang in week 1 ($F(1,67) = 4.386$, $p = 0.040$). Though there was no main effect of sex on song bout length ($F(1,67) = 2.518$, $p = 0.117$), there was a significant interaction between sex and week of observation ($F(1,67) = 4.635$, $p = 0.035$). All other interaction effects were not significant ($F_{sex*dose}(3,68) = 0.276$, $p = 0.842$; $F_{dose*week}(3,68) = 0.778$, $p = 0.510$; $F_{sex*dose*week}(3,68) = 0.549$, $p = 0.651$). Post-hoc tests revealed that the greatest dose differences observed were between the high dose versus control birds ($i\text{-}j_{control\text{-}high} = -3.777$, $p < 0.001$) and low dose versus control birds ($i\text{-}j_{control\text{-}low} = -2.915$, $p = 0.011$). Further analyses revealed that during week 1 of T-treatment, males sang longer songs than females ($F(1,33) = 11.254$, p

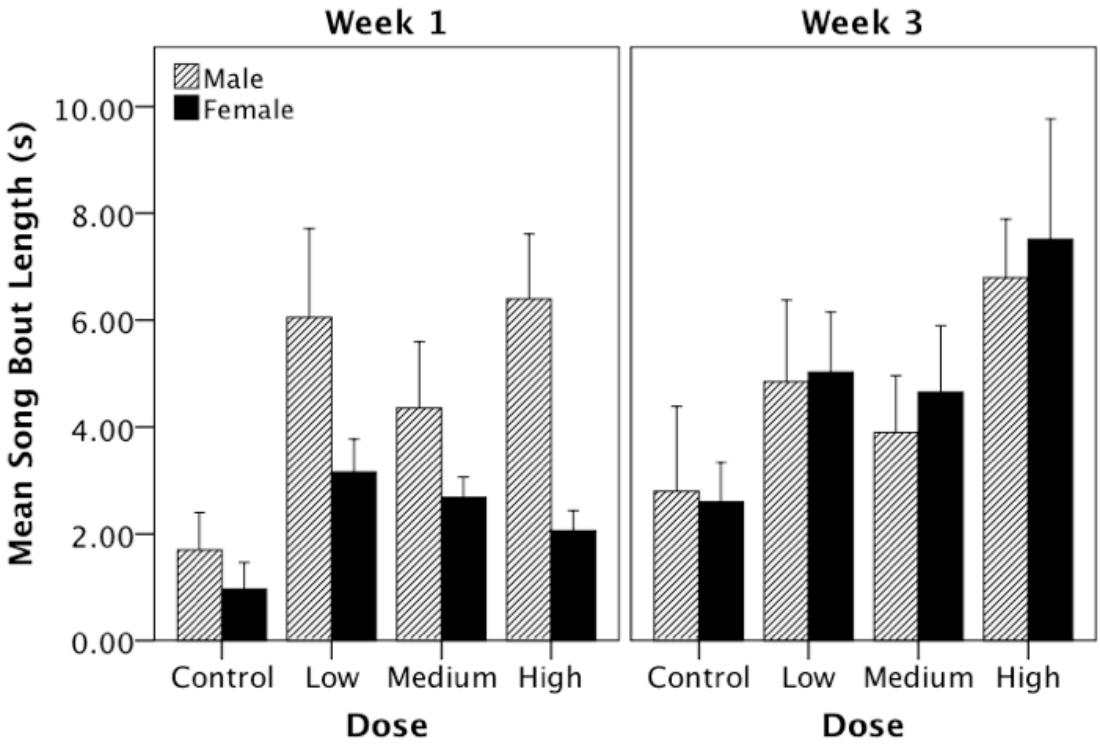


Figure 2.4: The effect of T-treatment on the song bout length (mean of the entire week) in males and females during week 1 and week 3. During the first week of T-treatment males sang longer songs compared to females, however, by week three male and female T-treated birds did not differ in song bout length.

= 0.002), however, this difference was not present during week 3 ($F(1,34) = 0.118$, $p = 0.734$).

Number of individual notes

As with song bout length, for the mean number of individual notes above amplitude threshold averaged across the entire week, there was a significant main effect of dose (see figure 2.5; $F(3,68) = 6.927$, $p < 0.001$). Furthermore, there was a significant main effect of week; birds sang more notes above threshold in week 3 of observation compared to week 1 ($F(1,68) = 7.268$, $p = 0.009$). In addition, there was a significant main effect of sex on the number of notes above threshold (i.e. males > females; $F(1,68) = 5.515$, $p = 0.022$), and importantly there was also a significant interaction between sex and week of observation ($F(1,68) = 4.235$, $p = 0.043$). All other interaction effects were not significant ($F_{sex*dose}(3,68) = 0.162$, $p = 0.922$; $F_{dose*week}(3,68) = 0.582$, $p = 0.629$; $F_{sex*dose*week}(3,68) = 0.966$, $p = 0.414$).

Post-hoc analyses revealed that the greatest dose difference was between the high dose versus control birds ($i\text{-}j_{control\text{-}high} = -31.002$, $p < 0.001$) and low dose versus control birds ($i\text{-}j_{control\text{-}low} = -25.365$, $p = 0.004$). There was a trend for the medium dose birds versus controls, however, after Bonferonni correction for multiple comparison the difference was not significant ($i\text{-}j_{control\text{-}medium} = -18.832$, $p = 0.069$). Furthermore, during week 1 of T-treatment, males sang more notes above amplitude threshold compared to females ($F(1,33) = 14.980$, $p < 0.001$), however, during week 3 of treatment this difference between males and females was no longer observed($F(1,34) = 0.031$, $p = 0.861$).

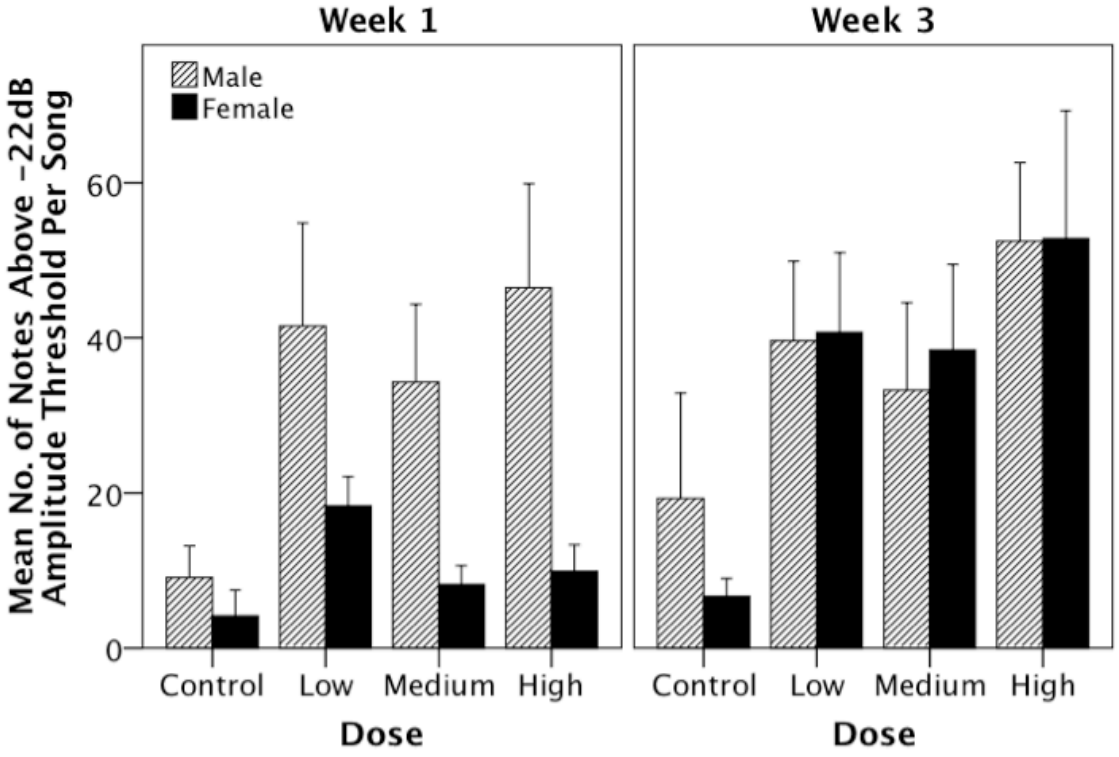


Figure 2.5: The effect of T-treatment on the mean number of notes per song (collapsed across the entire week) in males and females during week 1 and week 3. During week 1, male T-treated birds had more individual notes above amplitude threshold for acoustic measurement compared to females. This difference was not observed during week 3.

Mean vocalization energy

To further characterize the singing behavior of these birds, we also measured the mean vocalization energy; as previously stated, energy is a measure of the 'loudness' of a given vocalization (see figure 2.6). There was a significant main effect of dose on the mean vocalization energy ($F(3,68) = 3.235$, $p = 0.028$). In addition, there was a significant main effect of week; songs in week 3 of T-treatment were louder than songs in week 1 of T-treatment ($F(1,68) = 4.324$, $p = 0.041$). Furthermore, there was a significant main effect of sex; males tended to sing louder songs compared to females ($F(1,68) = 11.299$, $p = 0.001$). There were no significant interaction effects for the between subjects variables ($F_{sex*dose}(3,68) = 0.889$, $p = 0.451$; $F_{sex*week}(1,68) = 0.026$, $p = 0.871$; $F_{dose*week}(3,68) = 1.231$, $p = 0.305$; $F_{sex*dose*week}(3,68) = 0.544$, $p = 0.654$). Post-hoc tests showed that overall, birds treated with a high dose of T sang louder songs compared to controls ($i\text{-}j_{control\text{-}high} = -0.169$, $p = 0.027$).

We also found a significant main effect of day of observation within the week, i.e. the repeated measure variable ($F(6,408) = 15.172$, $p < 0.001$). In addition, there was a significant interaction between the day of observation and week of assessment ($F(6,408) = 10.108$, $p < 0.001$). Furthermore, there was a significant interaction effect of day of observation and sex ($F(6,408) = 6.868$, $p < 0.001$) and a significant three way interaction between sex, week, and day of observation ($F(6,408) = 7.312$, $p < 0.001$). There was also a significant four way interaction between day of observation, sex, dose, and week of assessment ($F(18,408) = 1.849$, $p = 0.044$; see figure 2.6). All other interaction effects of the within and between subjects variables were not significant ($F_{day*dose}(18,408) = 1.559$,

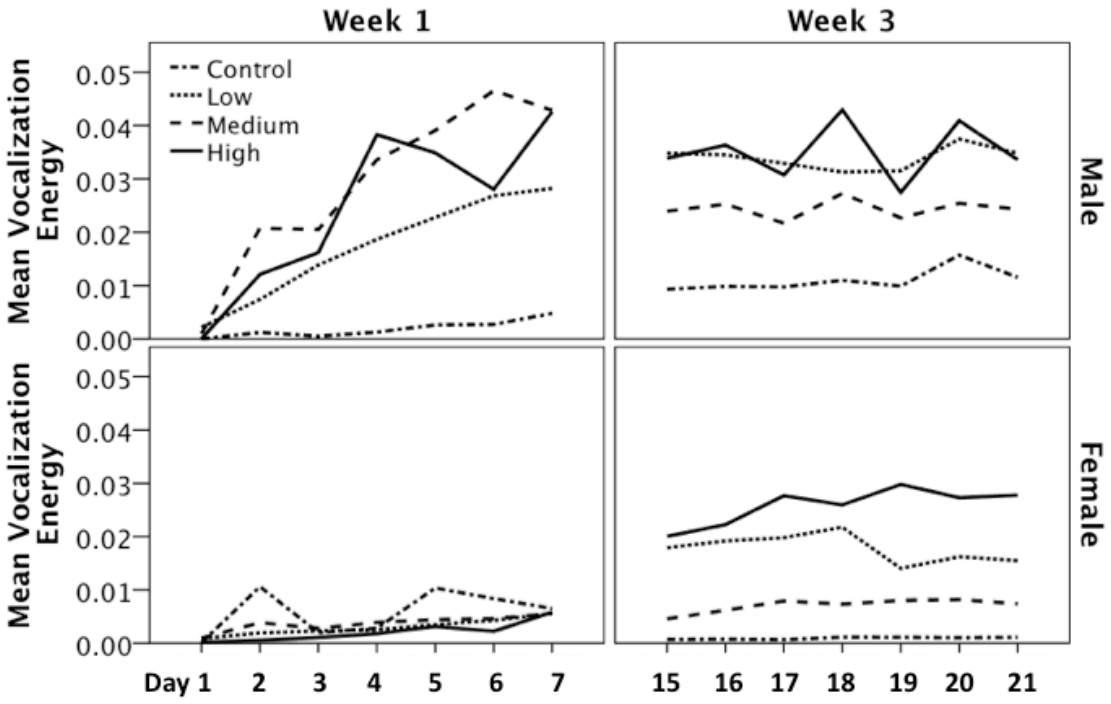


Figure 2.6: The effect of T-treatment on the mean vocalization energy in males and females during week 1 and week 3. Vocalization energy is the summed (integral) amplitude peaks of a given signal. During week one of T-treatment males sang much louder (i.e. greater energy) song compared to females. This difference was not observed during week three of treatment.

$p = 0.108$; $F_{day*sex*dose}(18,408) = 1.349$, $p = 0.195$; $F_{day*dose*week}(18,408) = 1.157$, $p = 0.316$).

Post-hoc analyses demonstrated that there was a significant effect of day of observation on vocalization energy during week 1 ($F(6,246) = 13.496$, $p < 0.001$), but not during week 3 ($F(6,246) = 1.898$, $p = 0.116$). Furthermore, a Bonferonni corrected MANOVA for days 1 through 7 (i.e. week 1 of treatment) revealed that males sang louder songs than females on days 3 through 7 of T-treatment ($F_{day1}(1,40) = 0.273$, $p = 0.604$; $F_{day2}(1,40) = 1.468$, $p = 0.233$; $F_{day3}(1,40) = 9.973$, $p = 0.003$; $F_{day4}(1,40) = 11.127$, $p = 0.002$; $F_{day5}(1,40) = 9.391$, $p = 0.004$; $F_{day6}(1,40) = 11.575$, $p = 0.002$; $F_{day7}(1,40) = 16.619$, $p < 0.001$). A separate Bonferonni corrected MANOVA showed that males and females did not differ on any day of treatment in week 3; however, there were non-significant trends on days 15,16, and 20 ($F_{day15}(1,40) = 3.840$, $p = 0.057$; $F_{day16}(1,40) = 3.240$, $p = 0.079$; $F_{day17}(1,40) = 1.385$, $p = 0.246$; $F_{day18}(1,40) = 2.841$, $p = 0.100$; $F_{day19}(1,40) = 1.553$, $p = 0.220$; $F_{day20}(1,40) = 3.552$, $p = 0.067$; $F_{day21}(1,40) = 2.264$, $p = 0.140$).

Correlation of song rate/composition and vocalization energy

Multiple linear regression analyses revealed significant predictive relationships between the acoustic structure of song and rate of song production with vocalization energy. In particular, during week 1 of T-treatment males who tended to sing songs with high entropy variance tended to also produce songs with greater energy (see figure 2.7A; $R^2 = 0.390$, $\beta_{standardized} = 0.533$, $\mathbf{\Delta}F(1,19) = 12.144$, $p = 0.002$). This finding was not observed for females during week 1 of T-treatment ($\beta_{standardized} = 0.086$, $p = 0.737$).

Likewise this finding was not observed during week 3 of T-treatment for males or females ($\beta_{\text{standardized Male}} = -0.249$, $p = 0.549$; $\beta_{\text{standardized Female}} = 0.374$, $p = 0.147$).

Furthermore, there was a distinct sex difference that emerged during week 3 of treatment where it was found that in males the greater the vocalization energy the greater the mean total number of bouts per observation (see figure 2.7B; $R^2 = 0.504$, $\beta_{\text{standardized}} = 0.710$, $\mathbf{\Delta}F(1,18) = 18.273$, $p < 0.001$). Thus males who sang loudly sang a greater total number of songs per sampling period. This finding was not observed in females during week 3 of treatment ($\beta_{\text{standardized}} = 0.099$, $p = 0.596$). In addition, during week 1 of treatment neither males nor females demonstrated this relation between energy and the mean total number of bouts per recording ($\beta_{\text{standardized Male}} = 0.141$, $p = 0.328$; $\beta_{\text{standardized Female}} = 0.162$, $p = 0.546$).

Song learning: syllable development

As described in the methods, we tracked the development of syllables and cross-correlated binary template sonograms of high amplitude song elements produced on the final day of treatment with sonograms of high amplitude syllables produced on days 5, 10, 15, and 20. High amplitude syllables were randomly re-sampled for each individual day [i.e. days 5, 10, 15, and 20] prior to cross-correlation to control for selection bias. We then calculated the percentages of these syllables in which the sonogram correlation was 0.95 or higher. These data were then analyzed in a 3-way split-plot factorial ANOVA (i.e. 3-way mixed-design with 2 between [i.e. sex and dose] and 1 within subjects variable [i.e. sampling day 5, 10, 15, or 20]). These data were only collected from birds sacrificed at 3-weeks.

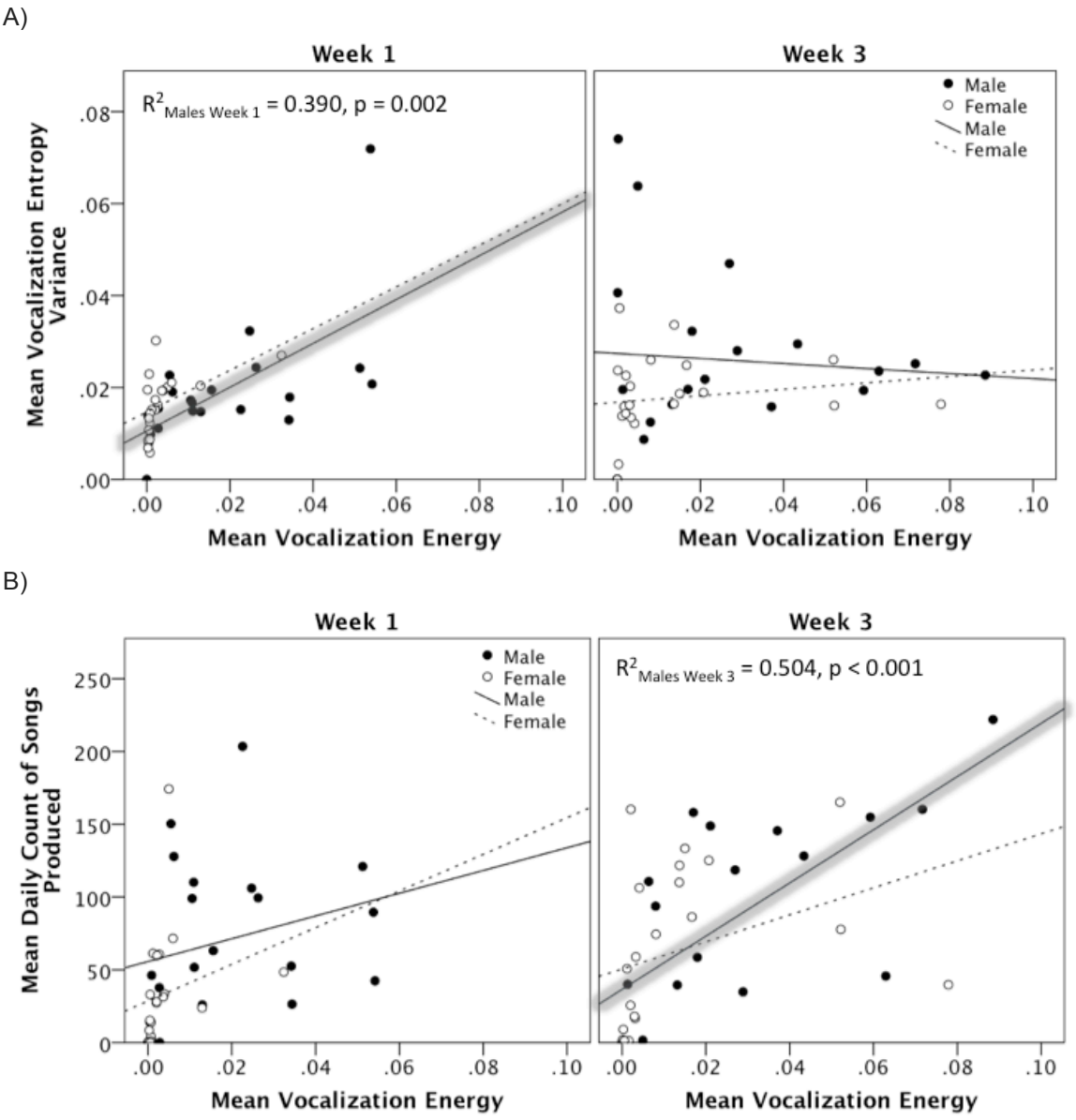


Figure 2.7: Correlation of mean vocalization energy with mean vocalization entropy variance and mean vocalization energy with the mean total song count in males and females during week 1 and week 3. Closed circles represent male mean values and open circles represent female mean values. Trend lines for males are solid lines and dashed lines for females. Significant trend lines are highlighted in grey. **A)** For males during week 1 there is a significant linear relationship between the loudness of a vocalization and its acoustic stability with louder vocalizations being having higher entropy variance, which is, associated with less stability (i.e. less stereotyped vocalizations). **B)** Furthermore, there was a striking sex and week interaction where for males only during week 3 we observed that the greater the vocalization energy the greater the mean total number of bouts per recording.

We found that there was a significant effect of sampling day on the mean percentage of high amplitude syllables above a r = 0.95 correlation threshold (See figure 2.8A; $F(3,102) = 16.037$, $p < 0.001$). There was no significant main effect of sex ($F(1,34) = 0.475$, $p = 0.495$). Unexpectedly, there was no significant main effect of dose ($F(3,34) = 1.748$, $p = 0.176$) despite the mean percentage of syllables above threshold remaining constant at approximately 10% for control birds across sampling days. However, there was an interaction between the sampling day and dose, but, after Greenhouse-Geisser correction for non-sphericity the trend did not meet criterion for significance ($F(3,102) = 2.049$, $p = 0.054$). All other interactions were not significant ($F_{sex*dose}(3,34) = 0.299$, $p = 0.826$; $F_{day*sex}(3,102) = 0.399$, $p = 0.718$; $F_{day*sex*dose}(9,102) = 0.413$, $p = 0.902$). Post-hoc trend analysis revealed a significant linear trend where syllables were more highly correlated as the duration of treatment increased ($F(1,38) = 32.590$, $p < 0.001$). Furthermore, for this measure there was a large amount of variance between individuals. This large amount of variance did not provide enough parametric space to fully elucidate the differences between the birds treated with T and the blank treated control birds. To account for significant individual variation in the effect of dose on this measure we combined the low, medium, and high groups and re-analyzed the data comparing T-treated versus blank controls (see figure 2.8B).

As with the first analysis we found a significant effect of sampling day on the mean percentage of high amplitude syllables above a r = 0.95 correlation threshold ($F(3,114) = 6.483$, $p < 0.001$). Likewise, there was no main effect of sex ($F(1,38) = 0.815$, $p = 0.372$). However, unlike the previous analysis there was a significant main effect of treatment where T-treated birds had a higher mean percentage of high amplitude

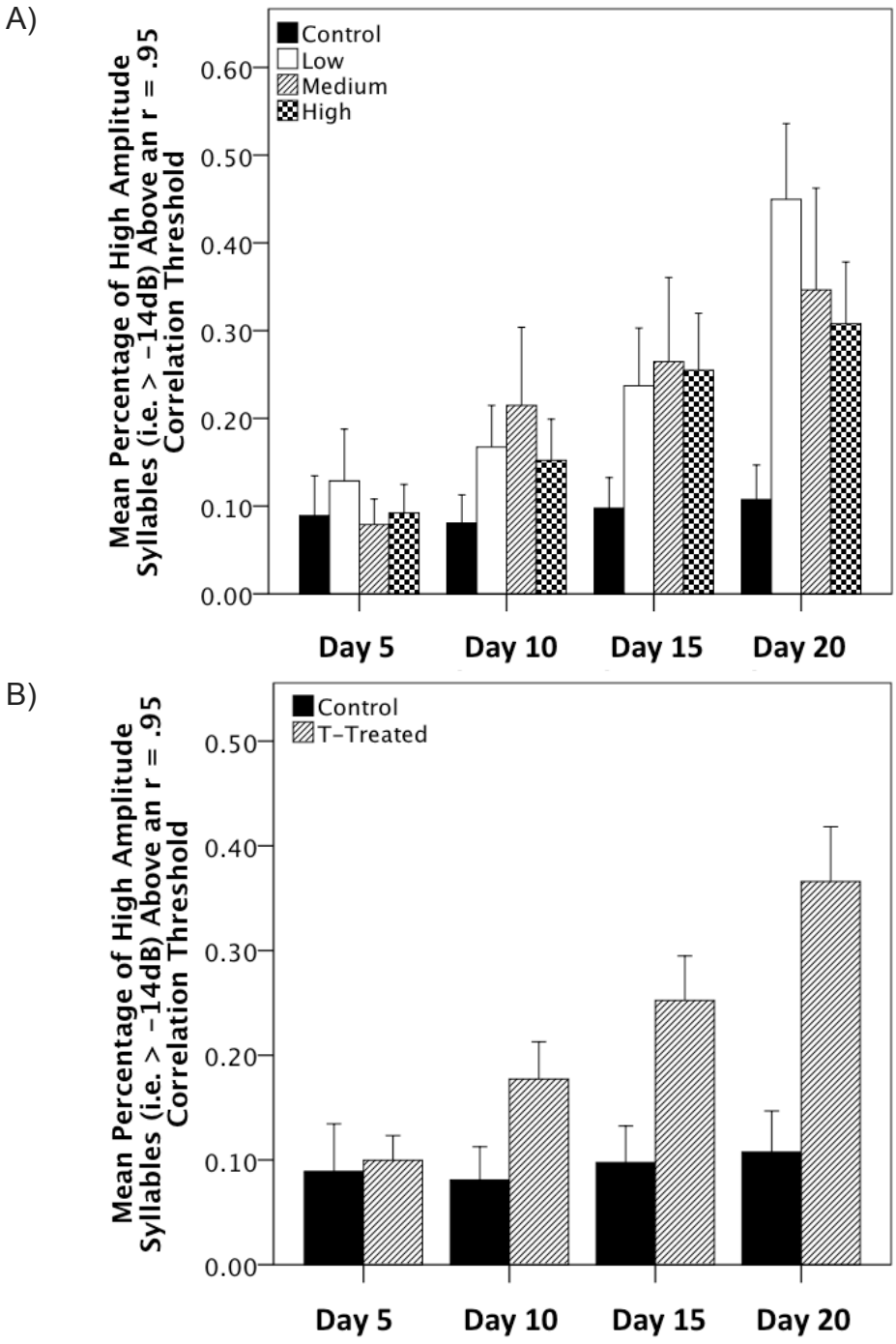


Figure 2.8: Mean percentage of syllables above a correlation threshold of r = 0.95. Across seasons male canaries drop and add new syllables to their repertoire (REF). This seasonal modulation of syllable development is regulated by cyclical changes in endogenous T (REF). Treatment with exogenous T induced singing and syllable development/refinement in both males and females. The pattern of syllable development did not differ between males and females. **A)** Furthermore, the pattern did not differ as a function of the dose of T received and **B)** when collapsed into T-treated versus blank controls it is clear that syllables become more stereotyped in the T-treated birds relative to controls.

syllables above an r = 0.95 correlation threshold ($F(1,38) = 5.279$, $p = 0.027$). There was no interaction between sex and dose ($F(1,38) = 0.230$, $p = 0.634$). However, there was a significant interaction between sampling day and T-treatment on the mean percentage of syllables above an r = 0.95 correlation threshold ($F(3,114) = 4.472$, $p = 0.008$). All other interactions were not significant ($F_{day*sex}(3,102) = 0.288$, $p = 0.806$; $F_{day*sex*dose}(9,102) = 0.566$, $p = 0.615$). A Bonferonni post-hoc test revealed that on sample day 5 there was no effect of treatment ($F(1,42) = 0.766$, $p = 0.387$), by day 10 there was a non-significant trend for T-treated birds to have a greater mean percentage of syllables above threshold ($F(1,42) = 3.088$, $p = 0.087$) and on days 15 and 20 this effect was significant ($F_{day15}(1,42) = 5.434$, $p = 0.025$; $F_{day20}(1,42) = 8.141$, $p = 0.007$).

Song control nuclei volumes

One of the fundamental aims of this experiment was to assess whether there are differential responses in the brain of males and females to a same range of T doses and how these changes relate to the behavioral changes we observe. As previously mentioned, we measured the volumes of the following song control nuclei: HVC (used as a proper name), Area X, and the robust nucleus of the arcopallium (RA; see figures 2.9A-C). We found that for all three song control nuclei there was a robust main effect of sex where males had larger nuclei volumes compared to females ($F_{HVC}(1,73) = 42.264$, $p < 0.001$; $F_{AreaX}(1,73) = 74.090$, $p < 0.001$; $F_{RA}(1,73) = 13.303$, $p < 0.001$). Surprisingly, however, the main effect of dose was not significant ($F_{HVC}(1,73) = 0.899$, $p = 0.448$; $F_{AreaX}(3,73) = 0.478$, $p = 0.699$; $F_{RA}(3,73) = 0.962$, $p = 0.417$). Though there was no significant main effect of week $F_{HVC}(1,73) = 0.058$, $p = 0.811$; $F_{AreaX}(1,73) = 0.797$, $p = 0.376$; $F_{RA}(1,73) = 1.919$, $p = 0.171$) there was a significant interaction of dose and week (F_{HVC}

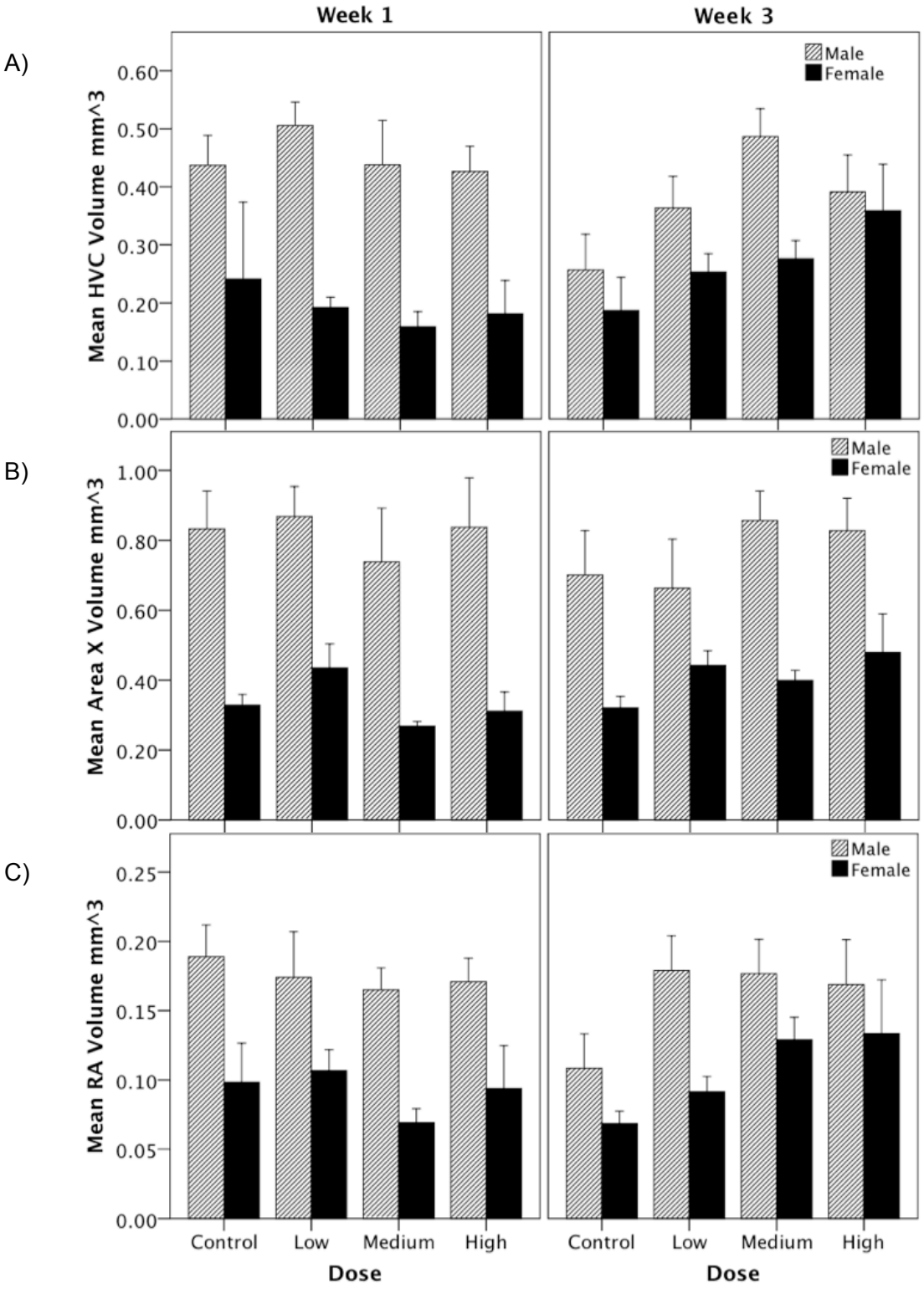


Figure 2.9: Song control nuclei volumes. Overall, males had larger volumes of song control nuclei for nucleus HVC (**A**), Area X (**B**), and the robust nucleus of the arcopallium (RA; **C**) compared to females. T also increased the volumes of HVC and RA on week 3 only.

(1,73) = 3.930, p = 0.013; F_{AreaX} (1,73) = 3.030, p = 0.037; F_{RA} (1,73) = 3.416, p = 0.023). The interaction of sex and dose was not significant (F_{HVC} (3,73) = 0.809, p = 0.494; F_{AreaX} (3,73) = 0.371, p = 0.774; F_{RA} (3,73) = 0.202, p = 0.894). The interaction of sex and week was not significant though there was a trend toward significance for HVC and RA (F_{HVC} (1,73) = 3.898, p = 0.053; F_{AreaX} (1,73) = 0.513, p = 0.477; F_{RA} (1,73) = 3.080, p = 0.085). The interaction of sex, dose, and week was also not significant (F_{HVC} (3,73) = 0.986, p = 0.406; F_{AreaX} (3,73) = 1.212, p = 0.314; F_{RA} (3,73) = 0.257, p = 0.856).

Post-hoc tests focusing on the dose by week interaction revealed that in brains collected at the end week 1, there was no significant main effect of T (F_{HVC} (3,34) = 0.986, p = 0.415; F_{AreaX} (3,34) = 2.600, p = 0.074; F_{RA} (3,34) = 0.571, p = 0.639). However, in brains collected at the end of week 3 the main effect of dose was significant for HVC and RA, but not for Area X (F_{HVC} (3,39) = 4.101, p = 0.015; F_{RA} (3,39) = 3.898, p = 0.018; F_{AreaX} (3,39) = 1.345, p = 0.278). Further tests showed that the effect of dose was most significant in the comparison of the medium dose versus control (HVC: $i\text{-}j_{control\text{-}medium}$ = 0.196, p = 0.011; RA: $i\text{-}j_{control\text{-}medium}$ = 0.093, p = 0.018); the difference between high dose and control was not significant after Bonferonni correction for multiple comparisons though there was a statistical tendency for RA ($i\text{-}j_{control\text{-}high}$ = 0.075, p = 0.097).

DISCUSSION

Testosterone treatment and the induction of song

In this experiment, we investigated the activational effects of T-treatment in adult male and female canaries housed under the same photoperiodic conditions. We examined how efficacious varying doses of T-treatment were in modifying male and female song behavior, and how rapidly they affected this behavior and its quality. Serum testosterone concentrations increased in direct relationship with the size of the implant and were clearly sufficient to markedly increase the singing rate of castrated male and intact female canaries. All birds treated with T increased rates of singing relative to blank treated controls. Interestingly, however, birds treated with a low dose of T sang significantly more frequently than controls during week 1 and week 3 of observation. This finding is quite interesting given that birds treated with low dose of T did not display a statistically significant rise in circulating testosterone as compared to controls. One implication of these data is that that slight increases in T (increases so small that they are not distinguished by our assays) are all that is required to initiate the induction of singing. We also do not know if there has been a larger relative increase in T in the birds receiving the low dose as compared to the controls since we did not collect samples prior to treatment in this study.

Sex differences in T-induction of song

Although both sexes exhibited increases in the rate of singing and characteristics of song, these effects were usually associated with different response latencies in males and females. In the case of song rate, males responded with a shorter latency to T treatment: during week 1, males spent a greater absolute proportion of their time singing compared to females. However, by week 3 there was no difference between male and

females in the proportion of time-spent singing. Likewise, for the ratio of time spent singing versus calling (i.e. general vocalizing) there was a robust sex-based difference during week 1 that was no longer present during week 3. Furthermore, we see a similar pattern of response latency in the T-induced modulation of song characteristics, in this case, song bout length, number of notes, and energy (i.e. loudness). There was for example no significant difference between short and long term T-treatment (i.e. 1 vs. 3 weeks) on song bout length in males whereas this was the case in females. Furthermore, during week 1 of T-treatment, males sang louder songs with more syllable iterations (i.e. number of notes above detection threshold) compared to females; however, during week 3 of treatment this difference between males and females had disappeared.

These findings are quite intriguing particularly in light of the correlations that were identified in males only. Early in T-treatment males who sang loudly sang songs that were less stereotyped (i.e. high entropy variance) and later in T-treatment males who sang loudly tended to produce more songs in total. For male songbirds, song serves a dual function; mate attraction and defense display against other (typically male) conspecifics. T-treated males increased both rate and gain (i.e. mean energy) with short latency relative T-treated females; however this occurred prior to the refinement and crystallization of song as males who sang loudly sang less stereotyped songs (as noted by mean entropy variance). Additionally, males who sang at a high rate (i.e. high mean total song count) tended to do so with increased gain later in T-treatment (i.e. louder songs during week 3). These data demonstrate that T can activate behavioral plasticity in both males and females with differential patterns in the latency of response in correlation with specific

features of song. Longer exposure times to testosterone or perhaps supraphysiolgical levels of T (relative to naturally occurring male levels) may be required in females to immediately engage the full behavioral suite as expressed by male canaries early in T-treatment (i.e. week 1). However, it is also quite possible that T is acting by different neuroendocrine or neurochemical mechanisms in females relative to males and that the temporal discordance in latency we observe in the induction of song reflects this difference.

Song typically occurs in a reproductive or competitive context in male songbirds: it is simultaneously an inter-sexually attractive and intra-sexually aggressive vocal signal. In the transition from the photosensitive to photostimulated state, males show a quick and robust change in behavior: song rates increase, in territorial species boundaries begin to be demarcated, and general intra-sexual aggression also increases. In wild male canaries these changes can occur even before the onset of increasing day length given particular social and environmental contexts (Leitner, et al., 2003). These changes in behavior are related in part to changes (in this case increases) in gonadal steroid concentration and to the rapid response of males to these endogenous and exogenous factors (i.e. T and socio-environmental context; Leitner, et al., 2003). It is therefore advantageous, in terms of reproductive fitness, for male canaries to rapidly and robustly respond behaviorally to even slight increases in T. These selective pressures are presumably different in females who do not sing as much as males and whose song is under a different type of stimulus control. It is plausible that these actions of T on behavior have not been fine tuned during evolution in this sex.

Similarities in syllable development in males and females

To evaluate sensorimotor learning and the development of syllables, we cross-correlated high amplitude song elements produced on the final day of treatment with high amplitude syllables on days 5, 10, 15, and 20. We found that there was a significant main effect of sampling day on the mean percentage of high amplitude syllables above an r = 0.95 correlation threshold Surprisingly, however, there was no difference between males and females in the pattern. The overall pattern of syllable development was identical between males and females. Furthermore, there was no difference between the effects of the different doses of T given (i.e. low, medium, and high) on syllable development. When the groups were collapsed to T versus blank controls it was clear that T-treated birds exhibited a linear progression in the development of syllables with time, a progression that was not observed in control birds. There was thus clearly an effect of T on this song feature but the lowest dose was already fully effective for increasing song stereotypy.

Surprisingly, high amplitude individual syllable iterations became more stereotyped at the same rate in males and females indicating a similar pattern of T-induced syllable crystallization in both sexes. This finding stands in contrast to the sex differences observed in the increases of song rate and other song characteristics. T can thus affect some song features in a sex-specific manner whereas other features display similar responses following exposure to the steroid. This discrepancy points to the existence of divergent underlying mechanisms and possibly divergent selective pressures that organized the brain reaction to steroids in a sex-specific manner. It also supports our

previous suggestion (Ball, et al, 2008**)** that effects of T on the motivation and readiness to sing (reflected here in the song rate and probably song energy) are not necessarily mediated by the same mechanisms nor by an action in the same brain region as effects on song learning and song quality (measured here by syllable stereotypy).

Song control nuclei volumes

It was initially demonstrated that increasing serum T concentrations that are observed during the annual cycle correlate with increases in the volume of song control nuclei such as HVC, RA, and area X (Nottebohm, et al., 1987). Multiple studies later showed that treatment with exogenous T of photoregressed or castrated subjects of both sexes largely mimics this effect therefore demonstrating the causal role of T in this aspect of neuroplasticity (Sartor, et al., 2005; Smith, et al., 1997; Strand and Deviche, 2007). However, no study has to our knowledge directly tested whether identical treatments with exogenous T have identical effects on the morphology of the song-control system in both male and female canaries.

We found here that even after treatment with a range of doses of T there is still a robust sex difference in the volume of song control nuclei so that males have much larger volumes of HVC, RA, and area X than females. In this study, we failed to identify a significant overall effect of T on the volume of these nuclei. However, there was a significant interaction of dose and week resulting from the fact that by week 3, birds treated with T had larger HVC and RA (but not Area X) volumes compared to controls whereas such effects were not seen on week 1. An explanation for this unexpected pattern of results is suggested by the observation that in control males and females, the volume of

all 3 nuclei decreased, sometimes very markedly (e.g. for HVC and RA in males) between week 1 and week 3. This overall decrease in the volume of the song control nuclei may be the result of the birds being moved at the start of the experiment (at the beginning of T treatment) from group housing to individual cages in sound attenuated chambers. Placement into individual cages was implemented in order to obtain high quality sound recordings. Interestingly a previous study employing MRI that allowed investigators to measure within subject change over time in song nucleus volume found that RA volume decreased in female starlings after transfer for a group housing condition to an individual housing condition (van Meir, et al., 2004). Although not directly measured in the van Meir et al. (2004) study or in this study, housing the birds in isolation may cause an activation of the stress axis. There is evidence that corticosterone can decrease the volume of song control nuclei such as HVC, namely by inhibiting neurogenesis (e.g., Newman, et al., 2010) and it is possible that being placed in social isolation was perceived as a stressor by in the birds in our study. The effects of T that we were studying here could therefore be superimposed on a decreasing baseline in song nuclei volumes induced by the social isolation stress. Under these conditions, T did not produce an overall increase in volume of song control nuclei that has been reported in previous canary studies (Appeltants, et al., 2003; Nottebohm, 1980; Sartor, et al., 2005) but rather counter-acted the stress-induced decrease.

This interpretation cannot obviously be formally demonstrated since it is impossible to measure these volumes repeatedly by histology in the same birds but given that a similar finding was previously reported in starlings in which volumes of song

control nuclei were repeatedly measured by magnetic resonance imaging there is some support for this idea. In the previous study on starlings the stress likely associated with the transfer from the wild into laboratory conditions was similarly superimposed on the effects of exogenous T treatment. Since RA and Area X volumes were measured before and after treatments in the same birds it could be formally demonstrated in that study that volumes decreased in control birds while T blocked this decrease but was unable to produce a significant volume increase (van Meir, et al., 2004). The comparison of volumes on week 1 and 3 in this study suggests that a similar phenomenon was observed here. It must also be noted that if analyses are focused on week 3, the expected pattern of T action is observed.

Relationships between song control nuclei volumes and singing behavior

The comparison of these somewhat atypical volumetric changes with the behavioral effects described before leads to a number of very interesting conclusions. First, we demonstrate here that a) a major increase in song rate, vocalization energy and song stereotypy can take place in the absence of overall increases in the volume of HVC or RA (during week 1) and furthermore that b) these behavioral changes occurred for the most part while HVC and RA volumes were decreasing. These observations bring additional support to the notion that effects of T on singing behavior are taking place at multiple brain sites and in particular that the motivation to sing is likely controlled outside the song system, possibly at the level of the medial preoptic area (see Ball and Balthazart, 2008; Ball, et al., 2003; Riters and Ball, 1999).

Secondly, the present studies also demonstrate that many, but not all, aspects of the sex differences in singing behavior disappear, at various rates, when males and females are placed in a similar endocrine environment while at the same time sex differences in the volume of song control nuclei are maintained. This brings additional support to the notion that other brain areas must be involved in song control and/or that the volume of song nuclei represents a useful but incomplete measure of their functional potential.

The persistence of sex differences in volumes after 3 weeks of exposure to a similar endocrine environment additionally raises the question of the origin of these differences. In birds and mammals, sex steroids early in development (i.e. ontogeny) act to organize the brain in a sex-typical fashion and later in adulthood they act to activate these sex-typical pathways supporting the expression of sexually differentiated behaviors (Arnold and Gorski, 1984; Goy and McEwen, 1979; McCarthy, et al., 2012; Phoenix, et al., 1959). Although the sexual differentiation of the song control system remains poorly understood (Wade and Arnold, 2004) and there has been no work on this phenomenon in canaries (most work was performed on zebra finches; Arnold and Gorski, 1984; Adkins-Regan, et al., 1994; Wade and Arnold, 1996; and only one study on starlings; Casto and Ball 1996), female canaries are presumably not exposed to the same endocrine conditions during ontogeny as males. Female canaries were probably never exposed to high T concentrations before this experiment, contrary to males. It is therefore conceivable that previous exposure to T organized the brains of males to respond to T action in adulthood with acute sensitivity. It could also be argued that a 3 week-period was too short to

produce the full complement of T effects in the female brain and that the volumetric sex differences observed on week 3 only reflect a different rate of response so that females would after a longer period reach the same condition as males. There is however no indication in the available data that this would be the case (sex differences are in general as large on week 3 as on week 1) and only additional longer experiments could answer this question.

Alternatively, it has been demonstrated in zebra finches that volumetric sex differences in the song system are controlled in part by genetic sex differences that act independently of circulating concentrations of sex steroids (Agate, et al., 2003). The contribution of similar mechanisms is obviously conceivable, if not probable, in canaries and the sex differences in HVC, RA and Area X volumes observed here in T treated birds might reflect this phenomenon.

CHAPTER 3

IS TESTOSTERONE-TREATED ADULT FEMALE CANARY (SERINUS CANARIA) SONG AN EFFECTIVE BEHAVIORAL STIMULUS?

Singing in songbirds often occurs in a reproductive context; female songbirds hear the song of males and choose the male with the '*best*' song to mate (*best* being a highly subjective assessment relating to the female optimizing her selection based on her own quality and exposure to *different* males; Searcy and Nowicki, 2005; Nowicki and Searcy, 2005; Holveck and Riebel, 2009). Song quality in males is correlated with a number of factors that contribute to a males overall reproductive fitness; e.g. immune-competence, parasite load, life history, etc (Nowicki, et al, 1998; Duffy and Ball, 2002; Spencer, et al, 2005; Buchanan, et al, 2004). Song is a salient stimulus to female songbirds: song in and of itself can elicit sexual responses in adult females (Nowicki, et al, 2001, 2002). For example, in response to the playback of attractive male song female canaries perform a copulation solicitation display (CSD) which is indicative of sexual receptivity (Kruetzer, et al., 1994; Vallet and Kruetzer, 1995). Given this functional adaptation of song in birds there are robust sex-based differences in singing behavior and its neural substrate that can be observed. For temperate-zone songbird species males tend to sing more than females and have larger song control nuclei (Ball and Hulse, 1998). However, there is variation in the ability of sex-steroids, namely, testosterone (T) to modulate these sex-differences in adulthood (Harding, 2004).

For example, in canaries exogenous T radically changes the brain and singing behavior of females. In some key ways T appears to, at least partially, *sex-reverse* the

brain and singing behavior of adult females; song control nuclei increase in volume and song becomes more male-like (Nottebohm, 1980; Gahr and Garcia-Segura, 1996; Madison, Rouse, Jr, et al, *submitted*). The song of T-treated females is longer in duration, louder, exhibits greater stereotypy, and is more complex in composition compared to control females and males; they do not differ from T-treated males particularly if females were treated long term (e.g. 3 weeks; Madison, Rouse, Jr, et al, *submitted*; DeRidders, et al, 2002).

However, what is not altogether clear is if the song of T-treated females is generally perceived to be a masculine (i.e. attractive) conspecific signal. In other words, is the song of a T-treated female a salient enough stimulus to induce a sexual response in a reproductively active female listener? Furthermore, is there a similar level of gene activity in auditory areas specialized in the processing of conspecific song in response to the playback of T-treated female and male canary song?

We exposed estrogen treated adult female canaries to playbacks of T-treated adult female canary song, T-treated adult male canary song, and heterospecific song (Cassin's finch). We hypothesized that the mean CSD score elicited in response to playback of T-treated female and male song would not differ. Likewise, we hypothesized that both T-treated female and male song would elicit significantly greater mean CSD scores when compared to heterospecific song. Finally we hypothesized that amount of immunoreactivity of the immediate early gene zenk (also referred to as Egr1) would not differ between females who heard playbacks of T-treated female or male song but would be significantly lower in females that heard noise.

MATERIALS AND METHODS:

Animal Subjects and Hormone Treatment

Nine adult female American singer canaries (*Serinus canaria*) were obtained from a local breeder (Maryland Exotic Birds) and housed in an indoor aviary on an short day 8L:16D (light:dark) light cycle for a period of ten weeks. Birds were kept in 49 x 95 x 51 cm cages (three birds per cage) at Johns Hopkins University, Baltimore, MD and fed canary food and provided water *ad libitum*. After being on this photoperiod female ovaries were regressed and birds were photo-induced to a photosensitive state. The light schedule was then changed to long day-lengths (14L:10D) and birds were transferred to individual sound attenuated chambers (16" x 19" x 20"). This change in photoperiod induced a photo-stimulated state activating the reproductive axis. Within the individual housing chambers females were provided with a nest and nesting materials (strips of paper, cloth, and cotton).

To ensure that females were reproductively active and receptive to reproductive stimuli we then steroid primed the birds with a single 6mm subcutaneous Silastic implant (Dow Corning, Midland, MI, USA, no. 602-175; 0.76 mm inner diameter, 1.65 mm outer diameter) filled with 17β estradiol. Sexual receptivity was tested daily for a period of 3 weeks with playbacks of male song and presentation of stimulus males to the home cage. Behaviors such as copulation solicitation displays (which will be discussed further below) and number of copulation attempts were monitored. Furthermore, rate of egg lay and nesting behavior was monitored daily. Prior to experimental playbacks birds were held in silence for 1 week; egg lay and nest behaviors were monitored daily.

Playback design and Behavioral Analysis

Phase 1 – Does T-treated female song elicit a similar number of CSDs compared to male song?

27 song bouts from 24 individual adult T-treated canaries (n = 6 females treated with T for 1 week; n = 9 females treated with T for 3 weeks; n = 9 castrated males treated with T for 3 weeks) and 4 adult male Cassin's finches (i.e. hetero-specific song) were randomly sorted into groups of four for playback (e.g. male canary song, hetero-specific song, female 3-week T song, female 1-week T song). Playbacks occurred twice daily for a period of 5 days. The order of song-type was randomly assigned and birds did not hear a given order more than once nor did birds hear an individual song more than once. Furthermore, birds did not hear song bouts from particular individuals more than twice. Song bouts featured 4 individual songs with 10 seconds of silence between songs. Individual songs were matched for volume (average song bout amplitude = -18dB) and length (15 seconds). Between bouts of song there was 3 minutes of silence. The total time of song playback was 15 minutes.

During the song playbacks blind observers watched for the occurrence of the copulation solicitation display (CSD). The copulation solicitation display (CSD) indicates sexual receptivity. It is stereotypic suite of behaviors that facilitates copulation in canaries. As defined by Kreutzer and Vallet (1991) a complete CSD is when the responding female crouches, arches her back, bringing simultaneously the head back and the tail forward all the while vibrating the wings (which are separated from the body). Females, in response to the playback of male song, can perform CSDs readily in the laboratory. We measured the CSD score, which is the number of complete CSD's

summed for each song-bout type (i.e. male conspecific, female conspecific, etc.). To statistically analyze this measure we used a Friedman two-way analysis of variance by ranks for related samples with respect to alpha < 0.05 for significance.

Phase II – Does T-treated female song induce a similar pattern of ZENK expression compared to male song?

At the end of phase 1 birds remained in isolation housing for one week during which no song playback was heard. Birds were then played a 30-minute sample of 3-week T-treated male, 3-week T-treated female, or white noise. 90-minutes after the start of song playback birds were sacrificed and the brains were quickly extracted and fixed in 0.5% acrolein solution (19 ml 1M PBS and 1ml acrolein) for 2 hours at room temperature under agitation. The brains were washed four times for fifteen minutes each in 1M phosphate buffered saline (PBS) and then transferred to 30% sucrose solution overnight. Once saturated (noted by tissue sinking to the bottom of the sucrose solution), the brains were flash frozen in powdered dry ice for 5 minutes, then placed at -80°C until brain sectioning. Brains were sectioned in the coronal plane with a cryostat at 40μm thickness into three series and we collected every section from the rostral to caudal extent of the brain.

Brain Fixation and Histology

Tissue samples were processed in random order timing of procedure was similar across groups. Brain sections were washed in 0.1 M PBS three times, once in 1% sodium borohydride, then washed three times in 0.1 M PBS, once in 0.5% H2O2 for 15 min., then

the sections were washed three times with 0.1 M PBS. The sections were then incubated in the EGR1 antibody (1:2000, Santa Cruz Industries, SC-189; hereto and further referred to as ZENK) in 20% PBS/N at 4ºC for 48 hrs. The sections were then washed three times in 0.1% PBS/T, then incubated in biotinylated secondary antibody (goat anti rabbit IgG, 1:250) for 1 h, washed three times in 0.1% PBS/T, incubated in Streptavidin horseradish-peroxidase complex (Vector, 1:200) for 1 h, and then washed three times in 0.1% PBS/T. Antibodies were visualized by incubating the sections with the chromagen nickel-enhanced diaminobenzidine (Sigma Fast DAB) for 2-3 minutes.

Microscopy and Quantitative Analysis

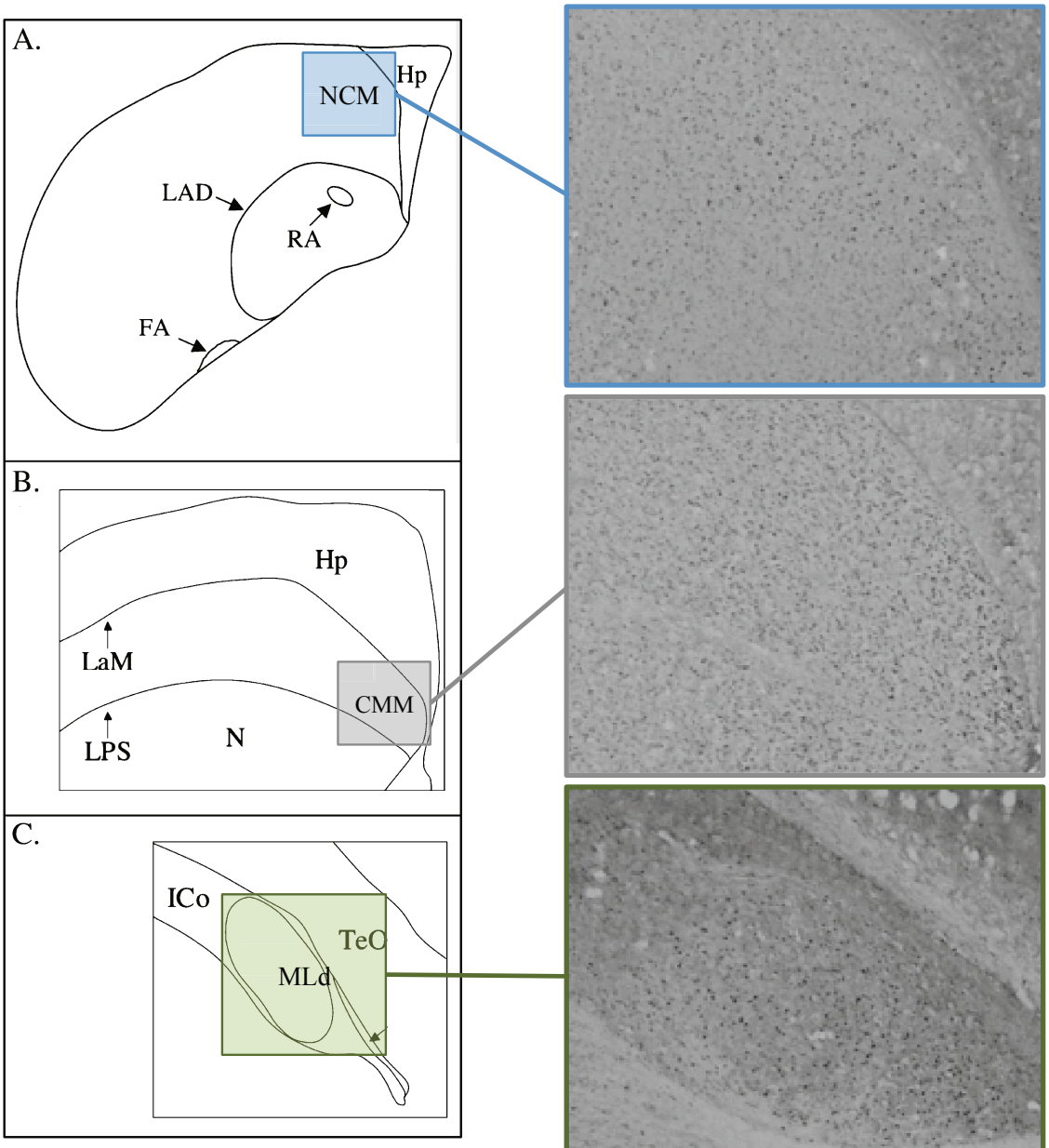
NCM, CMM and MLd were identified in coronal sections with reference to Stokes et al. (1974) and Maney et al. (2006). These regions were selected because previous literature clearly demonstrates that these areas have a many fold increase in ZENK induction in response to song playback (see figure 3.1; Maney, et al., 2006, 2007, 2008). Other auditory regions like Field L and the auditory thalamus do not display discernable differences in song-induced ZENK expression relative to controls (Mello & Clayton, 1994). For each region, ZENK immunoreactivity was quantified in three consecutive sections 120 μm apart for a single hemisphere. The quantified hemisphere was chosen at random except for instances when one side was damaged, in which case, the intact side was selected.

Images were acquired at 100x (NCM and CMM) or 50x (MLd) magnification using a bright-field light microscope (Zeiss Axioskop, Carl Zeiss, Thornwood NY). All images were approximately 350 KB. The light level on the microscope was set exactly the same

for each image; exposure time was automated for standard imaging. ZENK immunoreactive (ir) cell nuclei were quantified for each image using the thresholding feature in Image J (National Institutes of Health) as previously described by Maney and colleagues (2003). Threshold values were set manually for each individual image to optimize the agreement between blind observations and automated values. The number of particles with an optical density greater than a threshold value was counted within a predetermined area for each region of interest. For NCM, labeled cell nuclei were counted inside a 0.4-mm^2 box placed as close as possible to midline, adjacent to the hippocampus (see figure 3.1A). Labeled ZENK-ir cells in CMM were quantified with the following protocol; a 0.5-mm^2 box was placed approximately 400 um from the midline at the level where the lamina arcopallialis dorsalis (LaM) meets the lateral ventricle (see figure 3.1B). The area of Hp contained in the image was removed from the image before ZENK-ir cells were quantified with image J. MLd was traced in the three sections where it was largest and its borders most obvious with respect to the nucleus intercollicularis (ICo) and surrounding tissue (see figure 1C). We calculated the number of labeled nuclei per unit area sampled. To statistically test this measure we used a mixed ANOVA with playback stimulus as the between-subjects factors and the region of interest and section number as within-subjects factors. Significance was with respect to alpha <0.05.

RESULTS

Findings from phase I suggests that though female T-treated song induces a slightly greater response than hetero-specific song, T-treated male song yields a greater number of CSDs per session (mean = 4.75) than 3-week T-treated female (mean = 1.17), 1-week T-treated female (mean= 0.95) and hetero-specific song (mean = 0.29). One



Adapted from Maney, Cho, & Goode 2006
(Euro J Neurosci)

Figure 3.1: (A) NCM, (B) CMM, and (C) MLd are three auditory regions commonly assessed in songbird playback studies. These regions of interest were quantified in the coronal plane of section. Abbreviations: CMM, caudomedial mesopallium; FA, tractus fronto-arcopallialis; Hp, hippocampus; ICo, nucleus intercollicularis; LAD, lamina arcopallialis dorsalis; LaM, lamina mesopallialis; LPS, lamina pallio-subpallialis; N, nidopallium; NCM, caudomedial nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum; V, ventricle.

female subject was removed from the final analysis (both phase I and II) due to scores over three standard deviations away from the mean in each category. We found a significant effect of stimulus type in the in the mean number of CSDs induced from playback; in particular, we found that male song yielded greater CSDs (see figure 3.2; $p = 0.036$).

In phase II of the experiment we wanted to ask the question of whether the pattern of ZENK induction following song playback was similar between males and females. As with the behavior, we found that male song yielded greater ZENK immuno-positive cells when looking at the marginal mean values for NCM and CMM (see figure 3.3; $X_{CMM\ male} = 975.333$, $X_{CMM\ female} = 899.889$, $X_{CMM\ noise} = 765.000$; $X_{NCM\ male} = 1015.778$, $X_{NCM\ female} = 930.778$, $X_{NCM\ noise} = 823.000$). Importantly, however, these data have yet to be validated by an independent observer thus the current findings for the ZENK-immunoreactivity (ir) are only preliminary. Initial statistical analysis suggest that there is a significant main effect of playback type for NCM ($F(2,5) = 6.847$, $p = 0.037$). Male song yielded greater ZENK-ir compared to controls ($i\text{-}j_{male\ song\ -\ control} = 192.778$, $p = 0.014$); however, it did not yield significantly more ZENK-ir compared to female song ($i\text{-}j_{male\ song\ -\ female\ song} = 85.000$, $p = 0.128$). Surprisingly, no findings in CMM were significantly different ($F(2,5) = 0.835$, $p = 0.487$). As expected, however, MLd showed no statistical difference in ZENK-ir ($F(2,5) = 1.431$, $p = 0.322$).

DISCUSSION

For the female songbird, male song is a salient behavioral stimulus. In response to playback of male song, many female songbirds (like the adult female canary) initiate a

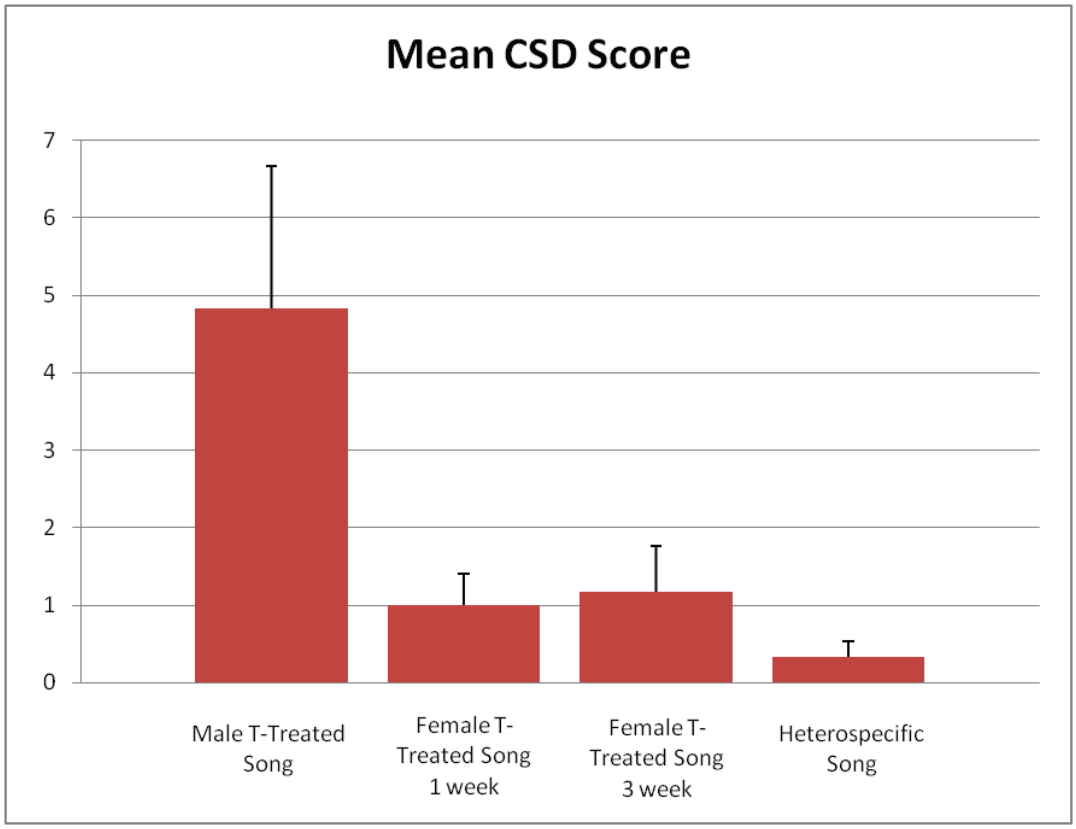


Figure 3.2. Adult female canaries preferred to respond to male conspecific song with CSDs, which is indicative of sexual receptivity. Surprisingly, however, females were able to respond somewhat to T-treated female conspecific song with CSDs despite this preference.

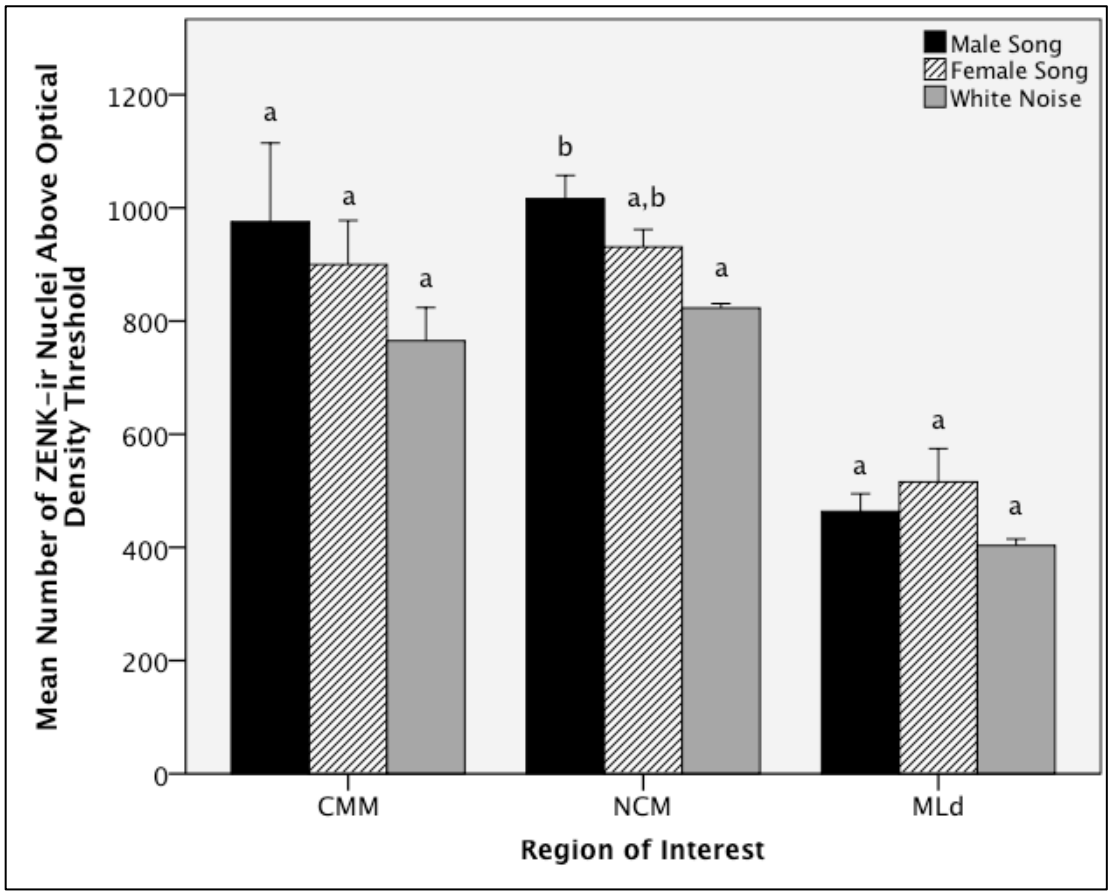


Figure 3.3. The overall trend of ZENK-ir followed what was observed with the behavior: in particular, T-treated male song yielded greater marginal mean values in NCM and CMM. However, only in NCM was this observation significant.

CSD. CSDs are indicative of the sexual receptiveness of a female and ostensibly her valuation of the stimulus quality (high quality song yields more CSDs compared to low quality songs; Leitner and Catchpole, 2002). Adult female canaries can sing spontaneously, however, their song is simpler and shorter compared to adult male song and lacks many species typical elements that are sexually salient (Vallet and Kreutzer, 1995; Vallet, Kreutzer, and Gahr 1996). Treating adult female canaries with T re-engages sensorimotor learning and the song masculinizes such that it is longer, more complex, and includes more behaviorally salient signals (Gahr and Garcia-Segura, 1996; Vallet, et al., 1996; Madison, Rouse Jr., et al., *submitted*). The current study asked if T-treated female song was an effective behavioral stimulus (i.e. capable of inducing CSDs).

We found that though T-treated adult female canary song was capable of inducing CSDs it was not as effective a signal as compared to T-treated adult male song. Likewise, we see a similar pattern in ZENK-ir, in particular, for NCM (these data have yet to be validated, thus all ZENK-ir results are preliminary). There are many ways to interpret this result, and a highly probably explanation was first shown in our lab some years ago (for reference see Sockman, Gentner, and Ball 2002). Previous experience with song modulates the perception of quality in mate choice cues (i.e. singing in songbirds; Sockman, et al., 2002). By being exposed solely to high quality male canary song for weeks prior to test we may have inadvertently biased responses to song playback. Though the song of T-treated adult female canaries sound and appear in spectrograms to be highly similar to male canary song, there may be subtle differences in the acoustic structure or arrangement of notes/ note-types that are not able to be perceived by human ears/ eyes. This argument is further supported by some sex-based difference in the correlates of song

quality and acoustic structure that we observed in the previous chapter entitled, *The effects of testosterone on vocal development and neural plasticity in adult male and female canaries (Serinus canaria): sex differences in song induction and measures of song quality* (for further discussion, refer to chapter 2).

CHAPTER 4

REPRODUCTIVE STATE MODULATES THE EFFECTIVENESS OF TESTOSTERONE TO INDUCE SONG BEHAVIOR IN ADULT FEMALE EUROPEAN STARLINGS, STURNUS VULGARIS

Singing in temperate-zone songbirds is controlled by photoperiodic, social, and environmental cues that interact with the birds own hormonal and neural control systems (Tramontin and Brenowitz, 2000; Catchpole and Slater, 2008; Kroodsma, 2004). For example, changes in photoperiod alone can cause increases in circulating levels of testosterone (T), singing, and volumes of song control nuclei in male European starlings (Bernard and Ball, 1995; Riters, et al, 2000; Dawson, et al, 2001; Stevenson, et al, 2010). As previously mentioned, the administration of exogenous T can induce an increase in singing and the volumes of song nuclei in male and some female songbirds independent of photoperiodic cues, indicating that photoperiod may act via T for many of its effects on song behavior and the underlying brain plasticity (Nottebohm, 1980; Hausberger, et al, 1995; Harding, 2004; see Schlinger and Brenowitz, 2002 for a review). There is evidence, however, that reproductive state can modulate T-induced behavioral and neural changes in mammals and birds (Campbell, et al, 1978; Ellis and Turek, 1983; Bernard, et al, 1997; Smith, et al, 1997). For example, data from male starlings suggests that being in a pre-breeding 'photosensitive' state increases the sensitivity to exogenous T on song control nuclei volumes, resulting in larger volumes of the song control nucleus HVC in photosensitive as compared to non-breeding 'photorefractory' birds treated with similar doses of T (Bernard and Ball, 1997). In addition, exogenous T in male song sparrows

(*Melospiza melodia*) is equally effective in inducing singing behavior despite individuals being in different reproductive state as a function of photoperiod (Nowicki and Ball, 1989; Ball and Nowicki, 1990). However, the effects of photoperiod on steroid regulation of song behavior and its relation to the effects on a variety of song control nuclei are not well understood.

European starlings are an excellent model system to study the modulatory role of photoperiod on T-induced singing behavior and song quality. Seasonality in starlings is distinguished by the fact that they display absolute photorefractoriness after being exposed to long day lengths for an extended period of time (Burger, 1947; Nicholls, et al, 1988; Dawson et al., 2001). This state of photorefractoriness is characterized by a regression of the gonads to a prepubescent-like state, as well as an inability to respond to long days of any type including photoperiods of constant day light (Burger, 1947; Dawson, et al, 1985; Falk and Gwinner, 1988; MacDougall-Shackleton, et al, 2009). When males are exposed to short day lengths for an extended period of time, the birds become sensitive (i.e., responsive) to the stimulating effects of long day lengths (Nicholls, et al, 1988; Dawson, et al, 2001).

Photoperiod is also involved in the regulation of adult female starling reproductive physiology; in general this regulation of female reproductive physiology is not related to changes in levels of T circulating in plasma (Dawson and Goldsmith, 1983; Dawson, 1984, 1997; Stevenson, et al, 2012). Furthermore, singing behavior in females appears to be at least partially regulated by photoperiod and is also independent of changes in levels of circulating T. Female starlings tend to sing in the non-breeding season when T is low and significantly reduce song output to almost nothing in the

breeding season when T is high (Pavlova et al, 2005; Pavlova et al, 2007a, 2007b). Nevertheless, adult female starlings can respond to exogenous administration of T by singing male-like songs despite not having measureable levels of endogenous T (Hausberger, et al, 1995; De Ridder, et al, 2002). This indicates that sex differences in song behavior in starlings are largely based on adult sex differences in circulating T rather than based on sex differences organized early in ontogeny (Arnold, et al, 1996; Wade and Arnold, 2004). Adult female starlings treated with T provide a unique model to investigate the activational properties of T independent of prior exposure to male-like concentrations of circulating T.

We used female starlings to study how the effects of T on singing behavior, song quality, and song system morphology are modulated by reproductive state. It has been assumed that T-induced changes in females are modulated by the same mechanisms as in males, but this has not been tested. We also hypothesized that photosensitive females will be more responsive to the effects of T and will exhibit an increase in singing behavior compared to photorefractory females. Finally, we hypothesized that the volumes of song control nuclei will be larger in T-treated photosensitive female starlings compared to photorefractory females.

METHODS

Animals and Photoperiodic Treatments

Fourteen wild-caught adult female European starlings were used in this experiment. All birds were captured using a drop down V-trap in early March 2007.

Upon arrival in the laboratory, birds were group-housed and maintained on a natural photoperiod (8L:16D; lights on at 1200 hr EDT, lights off at 2000 hr). Shortly after arrival all birds were laparotomized and the gonads examined in order to confirm sex and assess reproductive condition. All birds were housed in groups on 8L:16D for 6 to 7 months before the start of the experiment to maintain a photosensitive state. Animal husbandry of the starlings was in accordance with guidelines published by the National Research Council (2010). All experimental procedures were approved by the Johns Hopkins University Animal Care and Use Committee and adhere to standards of the Society for Neuroscience.

The fourteen female starlings were randomly assigned to one of two photoperiodic conditions: 1) long-day photorefractory or 2) short-day photosensitive. Seven birds were transferred to group housing on a long-day photoperiod (16L:8D; lights on 0700 hr EDT, lights off 2300 hr EDT) and seven birds remained in group housing on a short-day photoperiod (8L:16D; lights on at 1200 hr EDT, lights off at 2000 hr EDT) to maintain a photosensitive state.

T-Implantation

Previous data has shown that housing starlings on long-days for a minimum of eight to ten weeks can induce photorefractoriness, as determined by gonad size, molt and beak score (Dawson and Goldsmith, 1983). After 10 weeks, both groups received a 10mm length Silastic capsule (1.47mm i.d., 1.96mm o.d.) containing crystalline T. Implants were inserted through a small incision (> 2mm) over the left flank subcutaneously. Previous work in our lab has indicated that adult female starlings do not

sing in individual isolation housing or in general laboratory conditions in the absence of exogenous T (unpublished observations). Therefore, a non-T-treated group was not included as we were primarily concerned with the effect of reproductive state on T-induced changes song and its neural substrate. Immediately following implantation, birds were transferred to individually housed test cages. Photoperiod was held constant for each individual bird. Birds were implanted for a total of 3 weeks and during that time behavioral measurements were taken daily.

Behavioral Measurements

Bird vocalizations were recorded daily using an electret microphone (Radioshack Model 33-3013) and digitized using a custom software package with 16-bit resolution and 44.1kHz sampling rate. Over the 3 week experimental manipulation, song samples were automatically recorded for a 2 hour period starting when the lights were turned on in the morning. Recordings were high pass filtered with a cutoff frequency of 900 Hz. The latency to sing post-surgical implantation of T-implant (in number of days) was measured for each bird. This is termed the latency score.

Song bouts were operationally defined as periods of at least 5 seconds of song with no more than 3 seconds of silence (see Bernard, Eens, and Ball, 1996). In addition, we measured the duration of each bout (in seconds). By measuring song bout length we have a first pass approximation of song quality/complexity. However, during the experiment some of the birds failed to sing during the 2-hr recording window. In addition, song production was more variable in the photorefractory group.

Refractory birds did not sing every day and there was a large day-to-day variance during the last two weeks of T-treatment. To control for these sporadic singing rates and provide a comparable number for each bird we used the peak singing day for each individual that sang to calculate mean song bout length (N=5, photosensitive; N=4, photorefractory).

The number of complete song bouts per sampling period was measured for each bird. Complete song bouts were defined as songs where at least 2 of the 4 phrase types characteristic of starling song were present. This measurement was tallied for each day of treatment. This modification was made to include the songs produced early in T treatment. To assess further the quality and complexity of the songs both a motif and features analysis were done.

Only birds recorded singing complete song bouts (i.e. songs where at least 2 of the 4 phrase types characteristic of starling song were present) were used for analysis. Not all birds sang complete song bouts. As a result only a subset of the birds were included in this analysis (Photosensitive N=5; Photorefractory N=4).

The motif analysis was used to estimate the repertoire size of individual birds. Sound analysis was done using Ravenlite (Cornell Ornithology Lab, Ithaca, NY) and each song (n=1,849) was visualized using oscillograms and spectrograms. Each unique motif/phrase was counted and categorized into one of four phrase types characteristic of starling song (whistle, variable/warble, rattle, and high frequency phrase types; Eens, 1997; see figure 4.1).

We also used Sound Analysis Pro (http://ofer.sci.ccny.cuny.edu) in our analyses of song structure. In particular, we measured Weiner entropy variance that is a

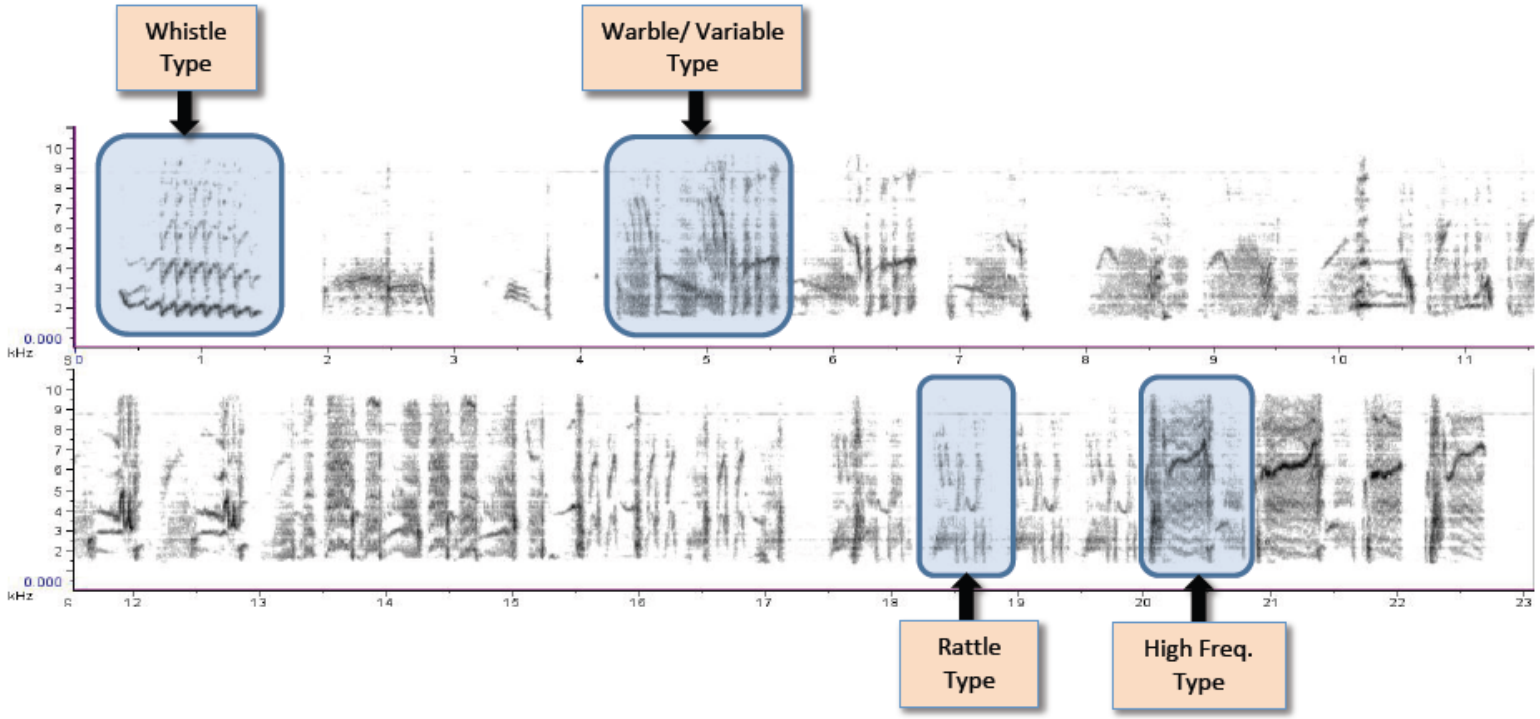


Figure 4.1: Starling song is a complex arrangement of harmonically variable vocalizations and though rich in acoustic diversity the various syllable phrases that make up starling song can be characterized into one of four motif or phrase types. The four phrase types are whistle, warble/variable, rattle, and high frequency phrase types.

quantitative measure of song stereotypy and generally shows an upward trend during song learning (Shank & Margoliash, 2009; Tchernichovski, et al, 2001). Weiner entropy is a measure of the spectral width and uniformity of an acoustic signal and the variance of this measure collapsed across a single bout of singing is indicative of the variability of that song (Tchernichovski, et al, 2001). In starlings high entropy variance tends to indicate songs with multiple syllable/phrase types, i.e. greater complexity (unpublished observations).

Peripheral Physiology

Beak color was assessed before and after hormonal implantation. In starlings beak color ranges from black to yellow (Hicks, 1934; Kessel, 1957). Bright yellow beaks indicate that testosterone is present in the blood while black beaks indicate that little or no T is available in the blood (Ball and Wingfield, 1987). The beak can therefore provide an independent indicator of hormonal condition. Beak scoring was as follows: 0 - completely black beak; 1 - 2/3 black beak with 1/3 yellow at the base; 2 – 2/3 yellow beak with 1/3 black toward the tip; 3 - completely yellow beak (Wydoski, 1964).

Radioimmunoassay

Blood samples were taken before T implantation and at the end of the experiment via puncture of the alar wing vein with a 25-guage needle. 300-500 µl of blood was collected into heparinized tubes at both sampling times. The blood samples were transferred into centrifuge tubes and centrifuged at 9000 rpm for 15 minutes. The plasma was removed and stored in vials at -20º prior to T assay. Plasma T concentrations were analyzed in a single run of duplicates (50µl) using a commercially available 125I Coat-A-

Count kit for total testosterone (Siemens Medical Solutions Diagnostics, Los Angeles, CA). This kit provides reliable hormone concentrations and has been validated for and previously used in starlings (Stevenson, et al, 2008; Stevenson and Ball, 2009; Cornil, et al, 2009). The antiserum is highly specific for testosterone (i.e. 100pg/ml) and shows negligible cross reactivity with other steroids including dihydrotestosterone (<3.5%); 17β-estradiol (< 0.01%); corticosterone (< 0.01%).

Perfusion & Peptide Immunocytochemistry

At the end of the experiment, birds were deeply anaesthetized with secobarbitol (50mg/ml IM) and transcardially perfused with heparinized 0.1M phosphate buffered saline (PBS) pH 7.5, followed by 4% paraformaldehyde. The brains were extracted and placed in 4% paraformaldehyde and left overnight at 4˚ C. The following morning, the brains were transferred into a sucrose solution (30% sucrose in 0.1 M PBS) and left overnight at 4˚ C. The brains were frozen with dry ice for two min., then left in a freezer (-70˚C) until sectioning. Brains were sectioned coronally (40 µm thick) using a cryostat.

Every third section was mounted onto gelatin coated slides and Nissl-stained with thionin. Adjacent sections were processed with immunocytochemical techniques for enkephalin (ENK). The boundaries of the forebrain nucleus lMAN and mMAN cannot be reliably discerned in Nissl-staned material but can be measured in ENK positive material (Ball et al, 1988; Bottjer and Alexander, 1995; Stevenson and Ball, 2010). However, the boundaries of HVC can be can clearly defined by both Nissl and ENK immunoreactive fiber staining (Stevenson and Ball, 2010).

The brains were processed in random order such that the time from tissue collection to processing was similar across groups. Once the brains were sectioned, the sections were washed in 0.1 M PBS twice, once in 0.5% H_2O_2 for 15 min., then washed three times in 0.1 M PBS and left overnight in normal goat serum (20% solution in 0.3% PBS/T [Triton X]) at 4ºC. Sections were incubated in primary antibody (1:2000 for ENK) for 24 hrs. In the morning the sections were washed three times with 0.1% PBS/T, then incubated in biotinylated secondary antibody (goat anti rabbit IgG, 1:250) for 1 hr, washed three times in 0.1% PBS/T, incubated in avidin biotin horseradish-peroxidase complex (Vectastain ABC, Elite Kit 1:200) for 1 hr, and then washed again three times in 0.1% PBS/T. The sections were then incubated in biotinylated tyramine (1:150 in PBS/T) for 1 hr, washed three times in 0.1% PBS/T, incubated in streptavadin horseradish peroxidase (1:200 in PBS/T) for 1 hr, and then washed another three times in 0.1% PBS/T. The antibodies were visualized by incubating the sections with diaminobenzidine (Sigma Fast DAB) for 6 minutes. Finally, sections were washed three times with 0.1 M PBS and mounted onto gelatin coated microscope slides. Sections were then serially dehydrated in ethanol and then placed in xylene for 5 min. The slides were then coverslipped using Permount (Fisher).

Song Control Nuclei Volume Reconstruction

Digital images of brain sections containing the region(s) of interest were captured using a microscope with a CCD camera connected to a computer. Digital images of the brain images were analyzed and the peptidergic-defined boundaries for each nucleus were traced using Openlab (Improvision® a Perkin-Elmer company). Volumes of each

nucleus were measured and normalized to the relative brain weight of the individual, which was measured immediately after brain extraction.

Statistical Analysis

We analyzed the data using a mixed design analysis of variance (ANOVA) unless otherwise noted. The relationships between measures were assessed with simple linear regression analyses. For the repeated measures variables, significance for main effects were corrected using a Greenhouse-Geisser correction for non-sphericity, contrasts were also corrected for non-sphericity using a Scheffe's S.

The number of song samples used for motif analysis varied dramatically between individuals and groups, which resulted in a multimodal distribution of songs per sampling period. Further, not all birds sang during the course of treatment, leaving unequal sizes for final motif analyses. This non-normal distribution of singing and unequal group sizes made parametric statistical analysis inappropriate. We used non-parametric statistics (Mann-Whitney U) to assess the difference in the number of unique phrases because equal sample size and normal distribution are not required for assessment of independent observations. All statistically significant results were evaluated with respect to $\alpha < 0.05$.

RESULTS:

We measured T in each bird before and after implantation: T-treatment significantly increased the concentrations of T in circulating plasma 3-weeks post-implantation in comparison to baseline ($F(1,12) = 27.65$, $p < 0.05$; see figure 4.2A). The T treatment appears to have been equally effective between groups, as we found no

significant difference in circulating levels of exogenous T between the photosensitive and photorefractory animals ($F(1,12) = 2.74$, $p = 0.124$). However, photosensitive females had significantly larger ovary mass on average compared to photorefractory females ($F(1,12) = 6.77$, $p < 0.05$).

Effects of T and reproductive state on beak scores

Exogenous T in male starlings is known to change the color of the beak from black to yellow (Goldsmith and Nicholls, 1984). We measured the beak scores of all female birds both pre- and post- T treatment. All of the birds, including both photosensitive and photorefractory females, started with black, low-score beaks. After application of T, the beak scores of photosensitive birds changed significantly over the three-week treatment period ($F(1,6) = 85.41$, $p < 0.05$), but the beak scores of photorefractory birds did not ($F(1,6) = 1.00$, $p = 0.356$). T-treated photorefractory birds were just starting to show signs of a change in beak color by the end of T treatment. We found a significant difference in beak scores between post-treatment photosensitive birds compared to either the pre-treatment measures, or when compared to the measures of photorefractory females ($F(1,12) = 66.15$, $p < 0.05$; see figure 4.2B). In short, reproductive state altered the effects of T on beak scores.

Singing behavior

The onset of singing in response to T-treatment did not differ between photosensitive and photorefractory birds (latency score, one-way ANOVA; $F(1,12) =$

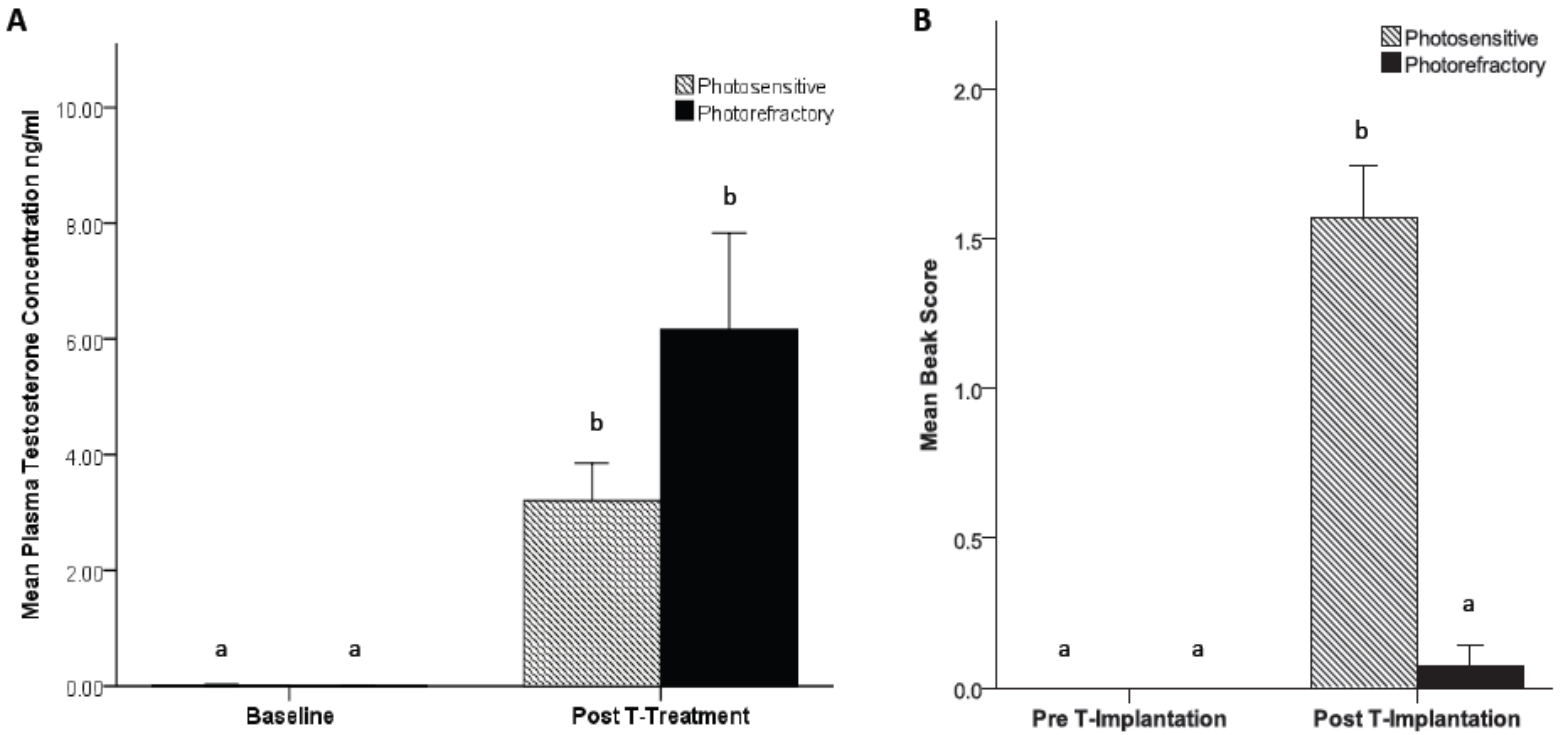


Figure 4.2: Black bars represent photorefractory birds. Striped bars represent photosensitive birds. **A)** T implants were equally effective in the two conditions as noted by baseline and end of study blood plasma concentrations; **B)** Beak score (which is indicative of circulating testosterone) differed by condition. T did not differ by condition on last day of study.

0.087, $p = 0.772$; see figure 4.3A). Birds started singing as early as the second day post T-treatment whereas other did not begin singing until much later in the treatment period, i.e., the eighth day of treatment. The onset of singing was quite variable and some birds did not sing at all over the course of T-treatment.

The rate of singing (mean number of song bouts per recording period) did not change significantly over the treatment period in the photorefractory birds ($F(2,14) = 0.21$, $p = 0.670$; see figure 4.3B), but the rate did increase over the 3 week period for photosensitive birds ($F(2,14) = 4.90$, $p < 0.05$). Singing rate was similar between groups during week 1, but by weeks 2 and 3 singing rate differed significantly between groups with the photosensitive birds singing at a much higher rate ($F(2,14) = 11.84$, $p < 0.05$). We chose to measure the mean song bout length on the peak day of singing, rather than collapsed across the course of treatment, so as not to bias the result due to differences in song rate (i.e. greater degree of difference in sample size). The mean song bout length on the peak day of singing did not differ significantly between groups ($F(1,7) = 3.36$, $p = 0.110$; see figure 4.3C).

Starling song is composed of a highly variable number of syllables and syllable/phrase types arranged into either long or short song bouts. Long song is complex, male-typical, and is composed of a diverse array of syllable/phrase types. Given that photosensitive and photorefractory birds did not differ in mean song bout length we measured the relative repertoire size of birds to further assess the quality/complexity of songs. Photorefractory singers had a larger relative repertoire size compared to photosensitive singers (total number of unique phrases; Mann-Whitney U-test, $U(5,4) = 18.5$, $p < 0.05$; see figure 4.4).

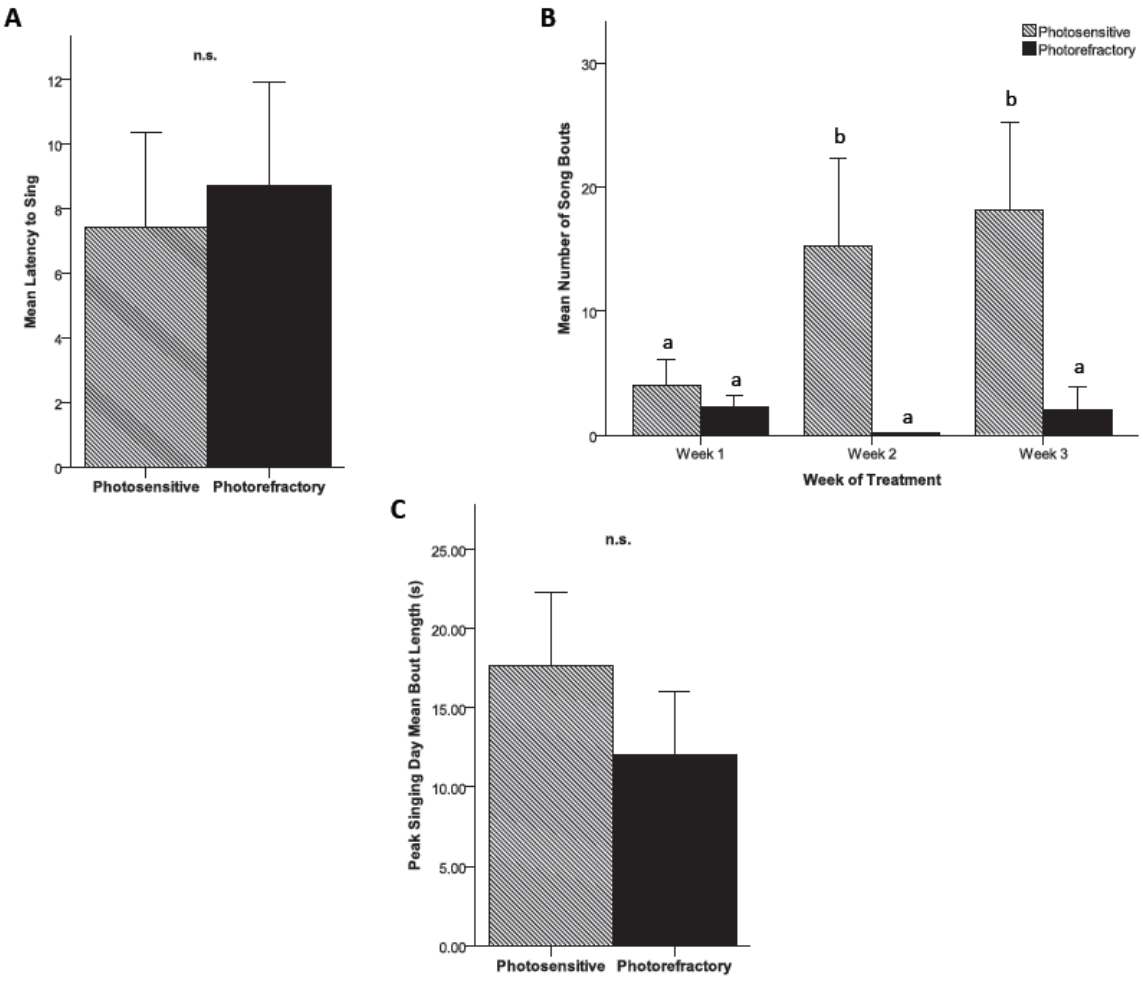


Figure 4.3: Black bars represent photorefractory birds. Striped bars represent photosensitive birds. **A)** Latency to sing did not differ across conditions. **B)** Rate of singing (noted by mean song bout number) differed by condition. Photosensitive birds sang at a higher rate than photorefreactory. **C)** Song bout length did not differ across conditions.

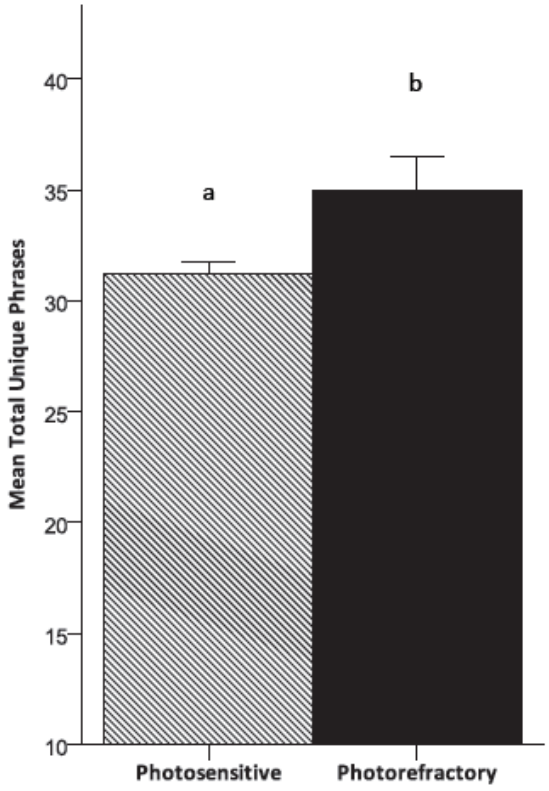


Figure 4.4: Photorefractory females had a relatively larger repertoire size when compared to photosensitive though they sang at a much lower rate.

During the first week of T treatment, we found no significant difference in the rate of singing between groups ($F(2,14) = 0.27$, $p = 0.630$). Because birds in both groups sang at similar rates during the first week, we looked for differences in the acoustic features of song during this period. We found that entropy variance changed significantly over the first seven days of T treatment (Greenhouse-Geisser correct repeated measures ANOVA, $F(6,36) = 3.62$, $p < 0.05$; see figure 4.5). Specifically, entropy variance increased significantly by day three of T treatment (the second day in which song was produced) and continued until day seven ($F(2,14) = 24.42$, $p < 0.05$). Day seven entropy variance was not statistically different from any previous day of treatment, however, it did approach significance ($F(2,14) = 6.74$, $p = 0.060$). Furthermore, there was a significant main effect of group; photosensitive birds sang songs with greater entropy variance than photorefractory birds ($F(2,14) = 13.20$, $p < 0.05$). The interaction of reproductive state and test day was not significant ($F(6,36) = 3.016$, $p = 0.074$).

Volumes of Song Control Nuclei

Many aspects of song behavior in songbirds are controlled by a forebrain circuit known as the song system. We measured the volumes of nuclei in this circuit and found no significant differences in the normalized volumes of these brain regions involved in song learning and production (Multivariate ANOVAs; HVC, $F(1,12) = 1.04$, $p = 0.329$; Area X, $F(1,12) = 0.87$, $p = 0.369$; LMAN, $F(1,12) = 1.11$, $p = .313$; RA, $F(1,12) = 0.02$, $p = 0.887$; see figure 4.6A & 6B). However, when non-singers (i.e. birds that did not sing complete song bouts) were removed from the analysis, we found the same result for all but one nucleus (HVC, $F(1,7) = 0.01$, $p = 0.921$; Area X, $F(1,7) = 0.07$, $p = 0.801$; RA,

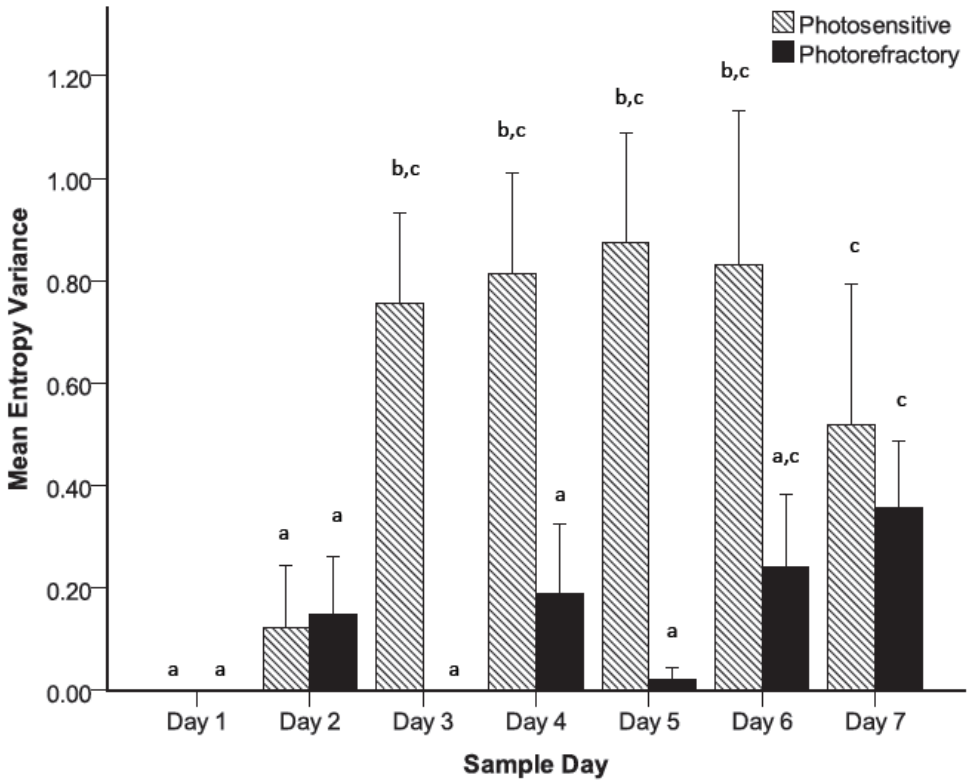


Figure 4.5: During the first week of treatment, when both groups were found to sing in similar rates, entropy variance changed significantly overtime and differed significantly by group. There was no interaction of time and group.

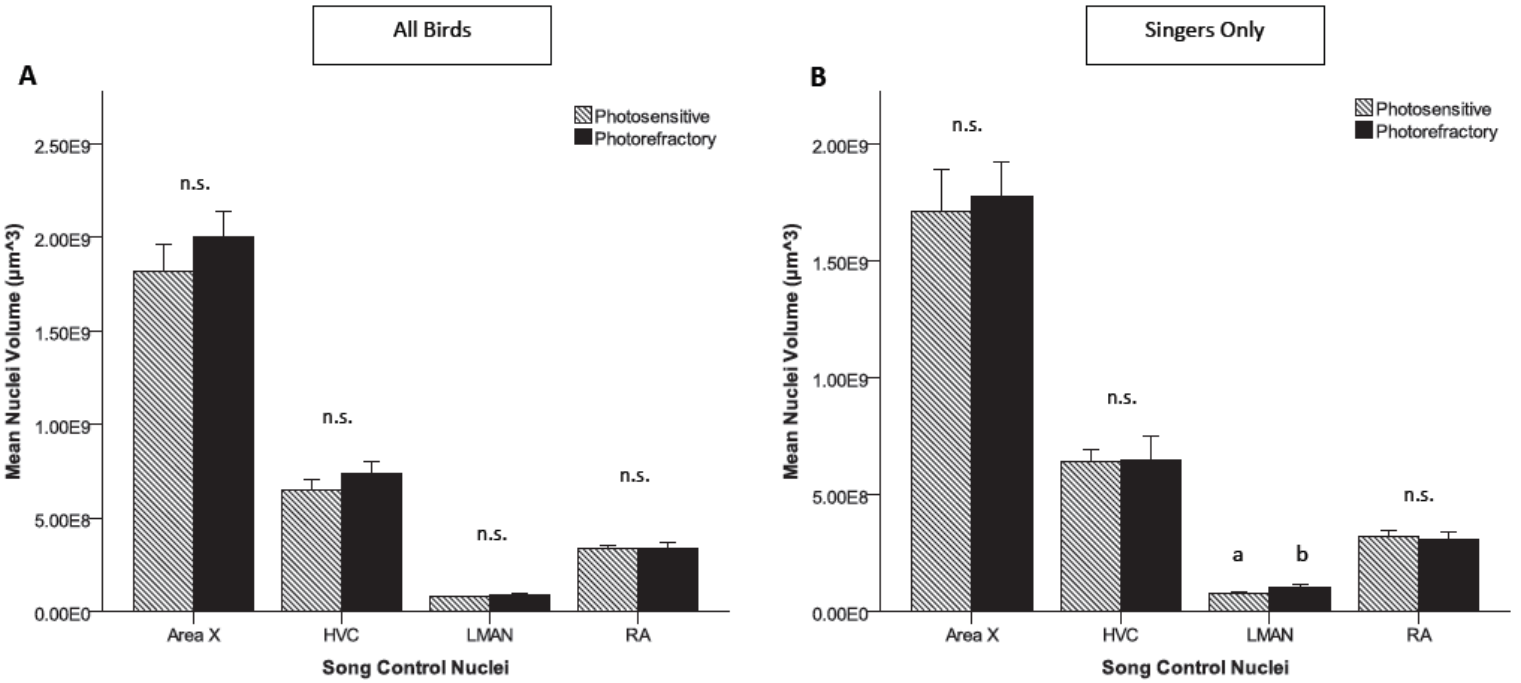


Figure 4.6: (A) Area X, HVC, LMAN, and RA did not differ significantly when all subjects were analyzed. **(B)** However, when only singers were included in the analysis, LMAN volume difference did cross the threshold for significance.

$F(1,7) = 0.18$, $p = 0.682$; refer to figure 4.6B). Photorefractory singers were found to have larger LMAN volumes than photosensitive singers ($F(1,7) = 5.78$, $p < 0.05$).

DISCUSSION

We exposed wild-caught adult female European starlings to two photoperiod regimens that resulted in the birds being in a photosensitive (i.e., pre-breeding or early breeding state) or photorefractory (i.e., non-breeding state) physiological state. We implanted birds from both groups with silastic capsules filled with T. Plasma T concentrations were higher three weeks post implantation as compared to pre-implant concentrations and there was no statistical difference between groups, indicating that the T filled capsules were equally effective in both groups. As discussed previously, beak color in starlings is a very sensitive indicator of the presence or absence of circulating androgens (Ball and Wingfield, 1987). Nevertheless, we found a significant effect of photoperiod on beak color scores: T-treated photosensitive females had significantly higher beak scores than photrefractory females.

In terms of behavioral measures, the onset of singing and the average song bout length on the peak day of singing did not differ between groups. However, photosensitive females sang at a much higher rate than photorefractory females over the course of T-treatment period. Photorefractory singers were found to have a slightly, but statistically significant, larger relative repertoire size as compared to photosensitive singers. Furthermore, during the first week of T treatment (when there was no group difference in song rate) we found that photosensitive birds sang more variable songs and therefore somewhat more complex songs. Weiner entropy variance was greater in

photosensitive females. As previously mentioned, Weiner entropy is a measure of the acoustic variability and complexity of a song (Shank & Margoliash, 2009; Tchernichovski, et al, 2001). Thus, it appears that song quality may be regulated by photoperiod in female starlings. However, the neural control of this difference in song quality and singing behavior is difficult to parse.

Our analysis, when it included all subjects in the study, did not reveal evidence that reproductive state modulated the effects of T on the song system volumes of HVC, Area X, LMAN, and RA. However, when non-responding females (i.e. birds who did not sing a complete song bout over the entire course of T-treatment) were removed from the analysis, we find that photorefractory singers had larger LMAN volumes as compared to photosensitive singers. This finding suggests a possible modulatory effect of photoperiod on LMAN volumes; however, these data were inconclusive.

Overall, our findings are consistent with the notion that being in a state of refractoriness generally reduces an individual's ability to quickly respond to exogenous steroid administration. As has been suggested in previous studies being in a post-breeding state is not only associated with a decrease in gonadal size and secretion but also with a change in tissue target properties and the ability to respond to T. In particular, being in a non-breeding state decreases T's ability to increase singing rate (latency to *start* singing was not different) and alter the acoustic structure and arrangement of song elements. More specifically, photosensitive females sang at a higher rate over time and sang songs that had greater acoustic variability than photorefractory females even though the relative number of unique phrases tended to be fewer in number. Photosensitive birds were more likely to include a diverse array of phrase types in an individual song bout. Thus the

compositions of song bouts were simpler in photorefractory birds despite the larger repertoire size resulting in a lower entropy variance during the first week of song.

Although we did not observe significant differences in brain region volumes, we did observe significant differences between the two groups on several measures of singing behavior (e.g. song rate and quality). It should be remembered that the song control system does not seem to regulate all aspects of song behavior (Ball, et al, 2008). Evidence from male starlings, for example, indicates that the rate of singing is regulated by the preoptic area (POA; Riters and Ball, 1999; Ball, et al, 2002). It may well be that in females song rate is controlled in a similar manner and that the POA of photosensitive females is more sensitive to the actions of T thereby increasing their rate of singing.

In addition to a difference in song rate we found a difference in the variability/complexity of song; photosensitive females had higher entropy variance. To account for this we propose that T changes the *state* of the neurons in the song control system changing the electrophysiological properties of the cells. This putative state change would result in a change in the probability of a neuron firing. Evidence from other songbird species supports this notion. In particular, there is data suggesting that T (and putatively its metabolites) play a role in the modulation of the electrophysiological properties of the song system across season (Meitzen, et al, 2009). It was found that the RA projecting neurons in the HVC of breeding condition male Gambel's white-crowned sparrows (i.e. long days plus T) had increases in the membrane time constant, capacitance, and evoked and spontaneous firing rate(s) relative to non-breeding controls (i.e. short days no T; Meitzen, et al, 2009). In addition, in adult female canaries it was found that exogenous administration of T increased the number of neuronal soma-

somatic gap junctions in HVC relative to blank-treated controls (Gahr and Garcia-Segura, 1996). Furthermore, androgens have been shown to hasten the developmental transition of N-methyl-D-aspartate– excitatory postsynaptic currents from slow to fast in the song control nuclei of male zebra finch; this finding is not observed in non-song control areas (White, et al, 1999). Taken together, these findings reinforce the notion that steroids alter the state of neurons by modulating neuronal excitability (see Zakon, 1998 for review)

Differences in neuronal excitability could also account for the observation that photorefractory singers had a larger repertoire of unique phrases. T is thought to recapitulate sensorimotor learning in adult females resulting in a masculinization of song (Hausberger, et al, 1995). It is possible that photorefractory singers had yet to actively engage sensorimotor learning. Sensorimotor learning is the dynamic modification of the acoustic features and arrangement of syllables/phrases that requires an active and intact song system.

Photorefractoriness may inhibit T-induced neuronal state change, essentially inhibiting the recapitulation of sensorimotor learning. This potentially means that the phrases incorporated into song may include vocal errors that are so different from other iterations that they could be mistakenly identified as unique. Across seasons males of a variety of species including starlings add new syllables to their repertoire and ostensibly experience a recapitulation of at least some aspects of sensorimotor learning (Nottebohm and Nottebohm, 1978; Samson, 1978; Bernard, et al, 1996). Thus, the difference observed in photosensitive and photorefractory female starlings may not actually represent a difference in vocal repertoire/ability but rather a difference in the stage of T-induced vocal development. Taken all together, the effects of reproductive state on T-

induced behavior were not related to differences in song nuclei volume but to putative actions in other brain regions and differential excitability of song system neurons.

CONCLUSION

We investigated whether T is equally effective in inducing changes in brain and behavior of adult female starlings in two different reproductive states. The data demonstrate that indeed reproductive state does moderate T effects in adult female starlings. But more generally, these data show that studies of female song production could shed new light on current views of neuroplasticity and neuroendocrine effects on vocal learning. By critically analyzing the changes that occur in response to T we can glean general principles about song behavior and its hormonal regulation. Elevating circulating levels of testosterone to male-like concentrations in females significantly changes song activity. Song becomes more male-like in structure and rates of singing increases. Song behavior changes can happen independently of volume changes to song control nuclei.

CHAPTER 5

LESIONS TARGETED TO THE ANTERIOR FOREBRAIN DISRUPT VOCAL VARIABILITY IN TESTOSTERONE INDUCED SENSORIMOTOR SONG LEARNING IN ADULT FEMALE CANARIES, SERINUS CANARIA

In the temperate zone, male songbirds typically sing more than females and there tend to be male-biased sex differences in the neural substrate (Ball, et al, 2008). These robust sex-based differences in the brain and behavior of temperate zone songbird species have often been accredited to the *organizational* effects of sex steroids (Arnold, 1996); i.e. steroids acting perinatally to organize the brain in a sex-specific manner (Phoenix, et al, 1959). However, in some species, such as canaries, testosterone (T) is able to induce male-like patterns of brain and behavior in adulthood (Nottebohm, 1980). In female canaries T treatment in adulthood alters induces growth in brain regions that control song learning and production, and appears to induce a recapitulation of the sensorimotor phase of song learning (Goldman and Nottebohm, 1983, Vallet, Kreutzer, and Gahr 1996). However, several song nuclei increase in size in response to T treatment (Nottebohm, 1980) and the contribution of particular song system nuclei to the different effects that T has on song behavior including the induction of the recapitulation of song learning is not clear.

The lateral magnocellular nucleus of the anterior nidopallium (LMAN) is a cortical-like song control nucleus, a component part of an anterior forebrain basal-ganglia feedback loop, and is directly implicated in song learning in juvenile male zebra finches (Scharff and Nottebohm, 1991; Aronov, et al, 2010; Fee and Goldberg, 2011).

Converging evidence from multiple research teams indicate that LMAN acts as an endogenous variability generator that serves to guide the trajectory of vocal motor learning in juvenile males (Kao, et al, 2005; Olveczky, et al, 2005; Thompson and Johnson, 2007). In adulthood, based on studies of male zebra finches, lesions to LMAN have more subtle effects on song production. As recently reviewed by Brainard and Doupe (2013) lesions to LMAN in adulthood do not have gross effects on song but variability is reduced if one does a careful rendition by rendition analysis of song or if one assesses adult song plasticity by a variety of learning tasks. What has not been investigated in detail is whether LMAN is essential for T induced plasticity in the case of adult canaries where T in females seems to result in a re-engagement of sensorimotor learning and the masculinization of song.

We individually housed adult female canaries, recorded their behavior prior to treatment, and then stereotaxic surgeries were performed (ibotenic acid lesions or sham controls). After recovery from the lesion-related surgery the birds were treated with T. We hypothesize that an intact LMAN is essential for T to be able to induce a re-engagement of sensorimotor song and induce the masculinization of female song. We also hypothexsize that the effects of T on the rate of song production would not be influenced by lesions to LMAN since both the POM and the HVC-RA-nXIIts pathway would be intact (Ball et al., 2008; Alward et al, 2013). We therefore predicted based on these hypotheses that prior to T-treatment these female canaries would not sing and that they would also not sing in response to lesion surgery. Further, we predicted that after

surgery and T treatment, females with LMAN targeted lesions would sing songs with less syllable variability, i.e. more stereotypy, as compared to sham control birds.

MATERIALS AND METHODS:

Animal Subjects and Experimental Design

13 Female border canaries (*Serinus canaria*) were housed on short-day length (8L:16D). After being held for a period of 6 weeks on this photoperiod birds were transferred to individual sound attenuated chambers (see figure 5.1). Birds were recorded daily for 90 minutes after lights on from the start to the end of the experiment. Birds were placed into individual sound attenuated chambers on day 0 and were held there for a period of 14 days before undergoing stereotaxic surgery for neurotoxic lesions (either saline sham or ibotenic acid lesion) targeted at the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Birds were given a period of 7 days to recover from surgery and to record immediate effects of LMAN lesion. Following this period, birds were then implanted (on day 21) with Silastic^{TM} capsules filled with T. Birds remained on T-treatment for 22 days and final behavior samples and tissue were collected on day 43 of the study.

Stereotaxic Surgery

Bilateral lesions of LMAN were based on stereotaxic coordinates modified from the canary atlas (Stokes et al., 1974). Birds were deeply anesthetized with isoflurane gas. The procedure was as follows: anesthesia was induced and maintained using an isoflurane gas (IsoSol isoflurane from Vedco. Inc, St. Joseph, MO) anesthesia system (Isotec 4 from SurgiVet, Inc., Waukesha, WI USA), fitted with a gas scavenging system,

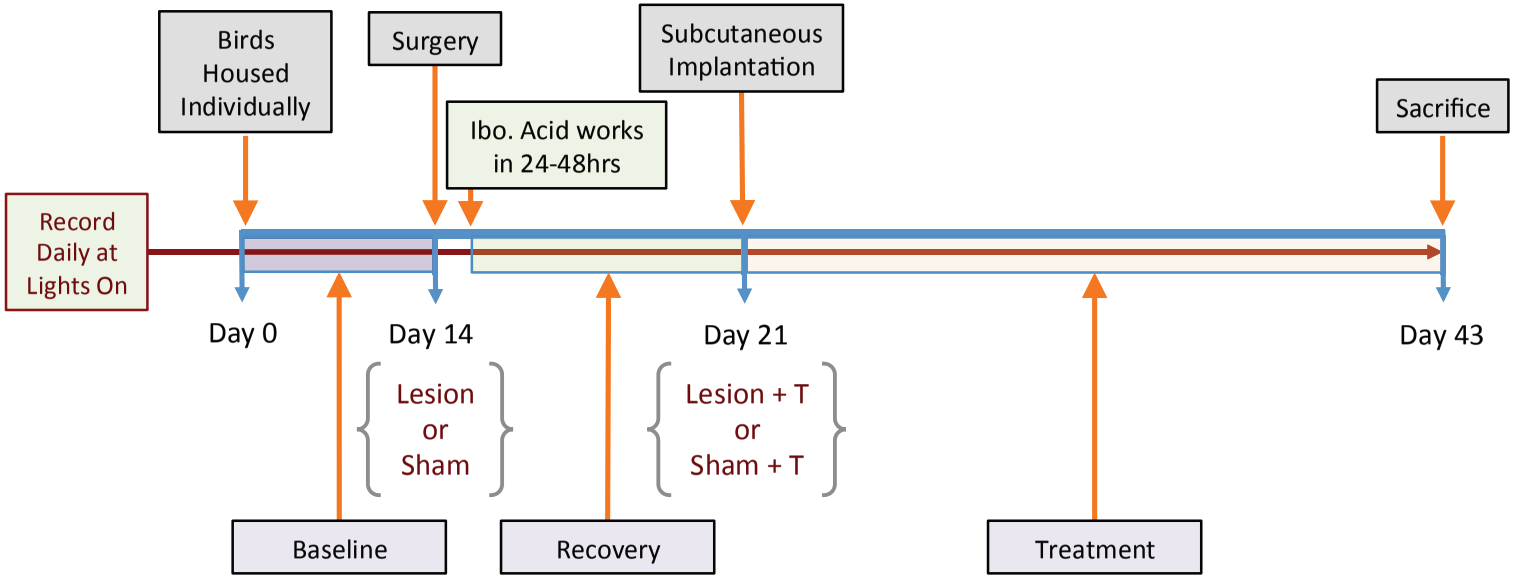


Figure 5.1: Experimental timeline. At the beginning of the study birds were transferred from group housing to individual sound attenuated chambers. They remained in these chambers for two-weeks. Birds then underwent stereotaxic surgery (lesion or sham control) and were given one-week to recover. Birds were then T-treated and remained on treatment for three-weeks. Behavioral data was sampled daily throughout the entirety of the study.

vented to a fume hood. LMAN was either neurochemically lesioned using ibotenic acid or sham lesioned with physiological saline. Lesions were done under a surgical microscope with a hamilton syringe connected to a microinjection unit. Ibotenic acid was dissolved in PBS at a concentration of 1 mg/mL. Approximately 0.25 µl of ibotenic acid and was delivered bilaterally to LMAN. Sham lesioned birds were be subjected to the same procedure but no drug was injected only saline. Coordinates were derived from pilot experiments of photosensitive female canaries. Initial pilot lesions were unilateral using the untargeted hemisphere as a control to locate the focal point and diffusion of chemical lesions. Later pilots were bilateral lesions to test acid volume effectiveness and survivability of the surgery. The coordinates used in this study were as follows: anterior-posterior – 4.25mm, medial-lateral – 1.43mm, dorsal-ventral – 2.40mm.

After birds were deeply anesthetized, an incision was made on the skin atop the head and retracted to expose the skull. Anterior-posterior, and medial-lateral coordinates were zeroed from the sinus, which was visualized through the birds' translucent skull; the zero point was marked on the skull with black ink. In cases where sinus was not visible, a portion of the skull was removed near the posterior portion to reveal the sinus and zero coordinates were taken from there. Small holes were then drilled through both layers of the skull at the points of entry. The holes were just large enough for the syringe needle tip to enter. Dorsal-ventral zero coordinates were then taken from the very top of the exposed meninges. A small tear was then made in the meninges by a sterile small gauge needle and the syringe was slowly lowered into position. Ibotenic acid was then slowly pressure injected slowly into the region of interest (total time approximately 5-minutes) and following that the syringe was raised. The hole in the skull was left unplugged as the

skin was then drawn together covering the points of entry. The wound was sutured shut with a sterile thin medical grade filament. Antibiotic ointment was applied around but not directly on the closed wound. The bird was then placed under a heating lamp and O2 was administered before return to the home cage.

Testosterone Implant

Birds were deeply anesthetized with isoflurane gas and a small incision is made (~1-2 mm) just above the right or left flank. Following incision, a metal probe was used to separate the skin from fat and muscle creating a pocket for the implant to rest. One 12 mm-long Silastic implant filled with T was then inserted. The skin was closed with tissue adhesive (Nexaband liquid bandage Veterinary products laboratory, Phoenix, AZ). This length of implant has been shown previously to be effective in inducing male-like concentrations of T in serum and a masculinization of song behavior (see chapter 2; Appeltants et al., 2003; Nottebohm, 1980; Sartor, et al. 2005).

Behavioral Analysis

Behavioral analysis followed what was previously done in chapter 2 of this text and was conducted as follows: sound files (converted from audio/video file format .mp4 to audio only .wav files) were sampled at 22050 Hz, which translates to a frequency range of 0 to 11 kHz. Audio files were highpass filtered with audio editing software Goldwave ^{TM} (version 5.55) set to a threshold of 900 Hz to remove low frequency noise (e.g. the sound of the fan/air vent and hum of the light). Sound spectrograms were created for each daily recoding using Avisoft SASlab (Avisoft Bioacoustics, Berlin, Germany). Spectrogram FFT (fast Fourier transform) lengths were set to 512 with an overlap of 75% for the temporal resolution. For each spectrogram the number of songs, song bout

duration (in seconds), number of calls, the total time spent singing and/or producing vocalizations (i.e. calls in addition to songs), and other acoustic features (i.e. mean entropy variance, fundamental frequency variance, and energy) for each recording were calculated and exported into an Excel spreadsheet.

Canary song has a characteristic acoustic structure and temporal pattern that distinguishes it from calls and we defined song as being bouts of vocalizations where the total duration was greater than 1.5 seconds of continuous syllables (featuring 5 or more syllables that have a peak amplitude value greater than -22dB) with inter-syllable intervals no longer than 500 milliseconds and a mean entropy value less than W = 0.550. One bird did not sing in response to T-treatment and was removed from the study.

The entropy variance and energy are features that have been previously discussed in chapters 2 and 4. The fundamental frequency variance gives a measure of variability of the lowest frequency in the individual acoustic signal or syllable. Fundmental frequency has lateralized neuromuscular control in canaries (Suthers, Vallet, Tanvez, and Kreutzer, 2004; Suthers, Vallet, and Kreutzer, 2012). Low fundamental frequencies are produced with the left syrinx and high frequencies by the right syrinx and in a single song the notes that compose canary song range in frequency from around 1 to 8 kHz (Suthers, Vallet, and Kreutzer, 2012).

To determine the stereotypy of song measures the coefficient of variation (CV = [SD/mean] x 100) was calculated for each bird for each day for each feature of song measured. The coefficient of variation is an established method to approximate the stereotypy of song features and has been used previously in studies of birdsong (Tramontin, Wingfield, and Brenowitz, 1999; Meitzen, Perkel, and Brenowitz, 2007).

We tracked the stereotypy of individual syllables in a manner similar to what was done previously in chapter 2. As previously mentioned, when song crystallizes, individual syllable iterations of a given type within a bout of singing and between bouts of singing become highly similar to one another (Waser and Marler, 1977; Marler and Peters, 1982). Crystallized song is characterized by high stereotypy (Marler, 1997). To measure the overall stereotypy of the syllables we isolated high amplitude vocalizations (peak amplitude > -14dB; song elements only, calls omitted) on the final day of recording, day 43, into individual .wav files. Syllables were then randomly re-sampled (maximum 100 syllables), categorized, and template sonograms were made for selected syllable iteration. High amplitude vocalizations (peak amplitude > -14dB; song elements only, calls omitted) on the days 21, 26, 31, 36, & 41 were then isolated into individual .wav files. Binary sonogram templates from the final day were then cross-correlated with sonograms for all song elements above amplitude threshold for days 21, 26, 31, 36, & 41 in Avisoft SASlab. The percentage of syllable correlations above an r = 0.95 correlation threshold was then tabulated for each bird for each day (i.e. days 21, 26, 31, 36, & 41).

On the final day of treatment, day 43, a syllable analysis was done to estimate the repertoire size of individual birds. Song samples for a given individual was aurally and visually inspected using spectrograms produced in Avisoft SASlab. Each unique syllable was marked and tabulated in a spreadsheet.

Finally, on the last day of treatment (day 43) we randomly resampled 10 individual songs and plotted template sonograms of the entire song sequence. Each song was then cross-correlated with one another in a correlation matrix using the Avisoft

correlator module (version 3) in Avisoft SASlab. The average correlation yielded from this matrix was then calculated. These procedures were done for each individual bird.

Histology and Lesion Quantification

At the end of the experiment, birds were euthanized by rapid decapitation and brains were extracted and placed in fixative (5% acrolein). Brains were slightly agitated in acrolein fixative for two hours, rinsed (4 x 15 minutes) in 0.01M Phosphate Buffer-Saline (PBS; pH 7.4) and cryoprotected in 30% sucrose until saturated. Brains were then flash frozen on dry ice and placed in the -70°C until processed for later analysis.

Brains were sectioned at 30 μm thickness using a cyrostat (Carl Zeiss) and were processed for NeuN immunoreactivity using the following protocol. NeuN is a neuronal marker that clearly and reliably delineates the boundaries of song control nuclei and yields comparable results to more traditional stains like Nissl (Phillmore, et al, 2006). Tissue samples were then processed in random order and the timing of procedure was similar across groups. Brain sections were washed in 0.1 M PBS three times, once in 1% sodium borohydride, then washed three times in 0.1 M PBS, once in 0.5% H2O2 for 1 hour, then the sections were washed three times with 0.1 M PBS. Sections were then incubated for 15 minutes in Avidin blocking solution (Vector laboratories; 1.5ml in 20ml 2% Normal goat serum in 0.3% PBS/T) then washed three times with 0.1 M PBS. Tissue sections were then incubated for 15 minutes in Biotin blocking solution (Vector laboratories; 1.5ml in 20ml 2% PBS/T-NGS) then washed three times with 0.1 M PBS. Sections were then incubated in the primary antibody for neuronal marker N (NeuN) in 20ml 2% Normal goat serum in 0.1% PBS/T at 4ºC for 48 hours. The sections were then washed three times in 0.1% PBS/T, then incubated in biotinylated secondary antibody

(Vector laboratories; goat anti rabbit IgG, 1:250 in 20ml 2% Normal goat serum in 0.3% PBS/T) for 1 hour, washed three times in 0.1% PBS/T, incubated in Avidin-Biotin horseradish-peroxidase complex (Vectastain ABC, Elite; 1:200 in 20ml 2% Normal goat serum in 0.3% PBS/T) for 1 hour, and then washed three times in 0.1% PBS/T. Antibodies were visualized by incubating the sections with the chromagen nickel-enhanced diaminobenzidine (Sigma Fast DAB) for 6-7 minutes. Brains sections were then placed in .01 M PBS solution and then mounted onto gelatin-coated microscope slides. The slides were open-air dried, rehydrated in 0.01M PBS and then serially dehydrated in ethanol at 30%, 50%, 75%, 95%, 95%, 100% for one minute each and a final step in 100% ethanol for five minutes. The slides were then cleared in xylene (Fisher Scientific) and coverslipped with Permount (Fisher Scientific).

Brain regions of interest were digitized using a bright field light microscope (Zeiss Axioscope, Carl Zeiss, Thornwood NY) with a CCD camera connected to a desktop computer. For each image, the area of the brain region was measured using NIH Image J. One sham control bird was selected at random as a reference to demarcate the boundaries of the lesioned portion of LMAN in the nidopallium. For the reference bird the volume of LMAN was reconstructed combining the areas of subsequent sections with the sampling interval (90 μm) using the formula for a truncated cone (developed by Smith et al., 1995). This method has been used previously in chapters 2 and 4 and has also been used previously in the literature (Bernard and Ball, 1995; Bentley et al., 1999; Bernard and Ball, 1997). For the sham control bird the nucleus was reconstructed for both the left and right hemispheres. Digital Images of each section containing LMAN were captured using a bright field light microscope (Zeiss Axioscope, Carl Zeiss, Thornwood

NY) with a CCD camera connected to a desktop computer. These images were then made translucent using NIH Image J and were overlaid on top of digital images of birds with LMAN targeted lesions taken from the same microscope and camera.

For these images landmarks such as Area X, MMAN, the relative position of the hyperpallium, and lateral most remaining labeled cells in LMAN were used to determine the sites of LMAN lesion reconstruction. In these composite images, the borders of the remaining portion(s) of LMAN were drawn and the area was measured. Next, the borders of the lesioned portion of LMAN was then drawn using the boundary provided by the reference image and the area was measured. Finally, the boundary of the lesioned area outside of LMAN was drawn and the area measured. Though the majority of lesion outside LMAN was confined to the nidopallium there were some cases where the rostral extent of lesion was tracked dorsally into small portions of the hyperpallium.

LMAN lesion volume was then calculated using the same formula as mentioned above. Surgery results were then categorized as lesion "hits" (n = 5), "misses" (n = 2), or sham controls (n = 5). LMAN targeted lesions were considered "hits" if and only if at least 50 percent of LMAN was lesioned in the combined average of the left and right hemispheres; they were otherwise considered "misses".

Statistical Analysis

To evaluate the song rate data we used a split-plot factorial analysis of variance (ANOVA; 2-way mixed-design with experimental day (day 1 – day 43) as the within and lesion target category [i.e. sham control, hit or miss] as the between groups variables). For these repeated measures variables, significance for main effects was corrected using a Greenhouse-Geisser correction for non-sphericity. Though conservative, this correction

assures that results are not influenced by variances that are potentially different between all possible pairs of groups in repeated measures ANOVA and has been used previously in chapter 2.

The mean values and coefficients of variation for the song features (entropy variance, fundamental frequency variance, and energy) and syllable stereotypy were evaluated separately for days 21, 26, 31, 36, and 41 of treatment. These days corresponded with the days in which the birds were exposed to T. To analyze each measure we used a split-plot factorial analysis of variance (ANOVA; 2-way mixed-design with experimental day as the within and lesion target category [i.e. sham control, hit or miss] as the between groups variables). For these repeated measures variables, significance for main effects was corrected using a Greenhouse-Geisser correction for non-sphericity. All post-hoc tests were corrected for multiple comparisons using Bonferonni's correction. The difference in pairwise comparisons and Bonferonni corrected p-values are reported for the effects of lesion target category and the polynomial trend analysis to generally describe the pattern of T-treatment response.

For the final day of singing, the mean number of unique syllables and mean song sequence correlation was tested in separate univariate ANOVAs. For the mean song sequence correlation the log transform was calculated to account for a skewed (i.e. non-normal) distribution of the data. All post-hoc pairwise comparisons were corrected for multiple comparisons using Bonferonni's correction.

Relationships (i.e. correlations) between the behavior on the final day of treatment (day 43) and lesion size (LMAN lesions and lesions outside of LMAN) were evaluated with two-tailed Pearson correlations. All results were considered statistically significant

for $\alpha < 0.05$. Effect size was calculated using partial eta squared values only for significant results.

RESULTS:

Testosterone Induced Increases in Song Rate

We tested for differences in the amount of singing over time which was measured by the proportion of time spent singing across baseline, recovery, and T-treatment, a total of 43 days. As expected we found a significant increase in the rate of singing in response to T-treatment; prior to subcutaneous implantation birds did not sing ($F(42,294) = 22.019$, $p < 0.001$, $\eta_{partial}^2 = 0.759$; see figure 5.2A). Also as expected, there was no effect of ibotenic acid lesions to the anterior forebrain on the rate of singing ($F(2,7) = 2.294$, $p = 0.171$) and there was no interaction of rate and lesion ($F(84,294) = 2.243$, $p = 0.140$). However, the complexity of the songs produced varied in observed sonograms by surgical treatment. Birds that received LMAN targeted lesions tended to produce songs that were simple, stereotyped in sequence and lacked syllable diversity (see figure 5.2B).

Acoustic features of song and measures of stereotypy/complexity

For the song feature and stereotypy analysis birds with LMAN targeted lesions were subdivided into two categories. As previously mentioned, “Hits” had 50% or more of LMAN damaged (n = 5) and “Misses” had less than 50% of LMAN damaged (n = 2). Despite the difference in syllable diversity anecdotally noted in the review of sonograms, the marginal mean values of the raw acoustic features of song did not differ between the sham lesion controls, LMAN lesion hits, and LMAN lesion misses (see table 1).

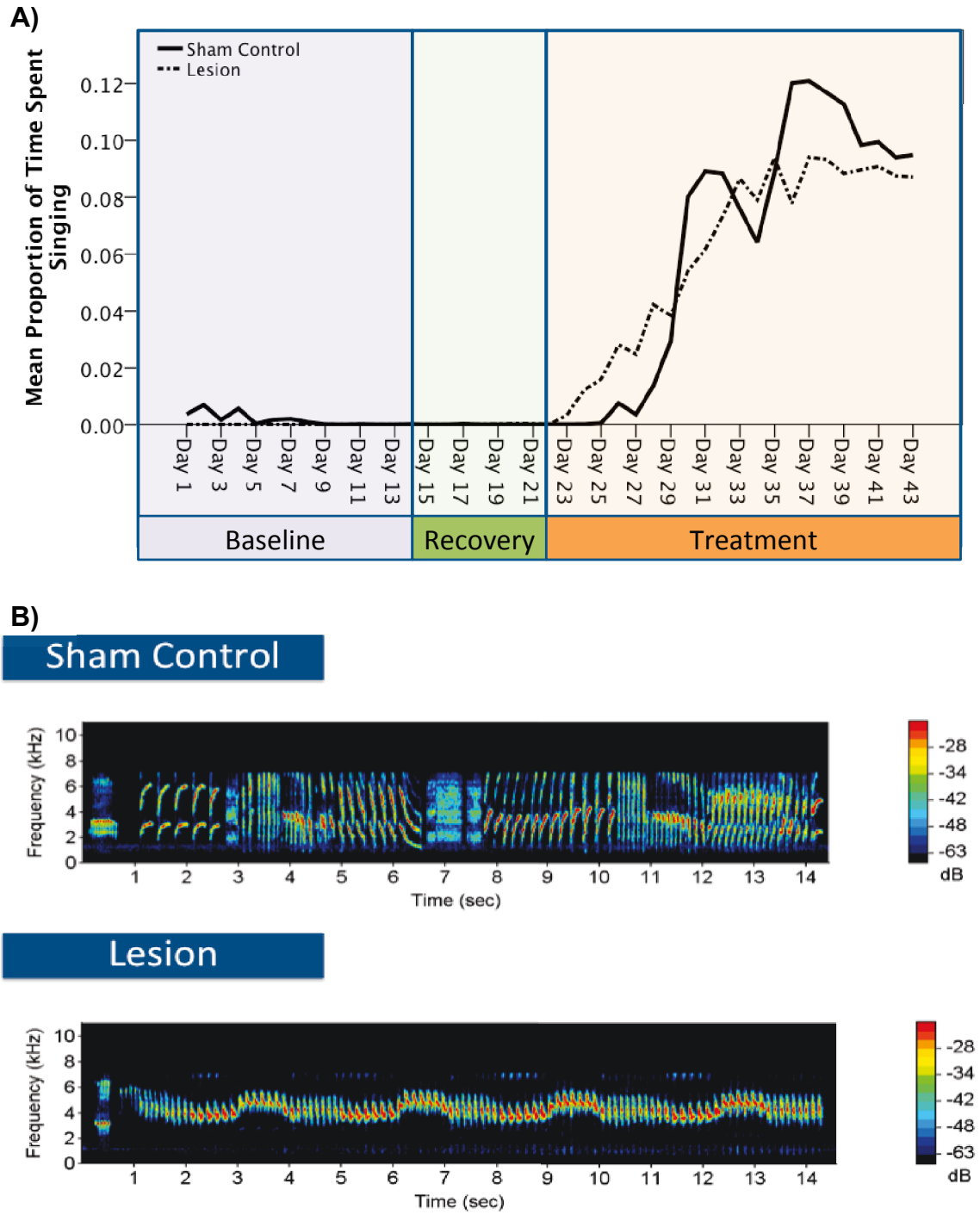


Figure 5.2: A) The effect of T-treatment on song rate. Prior to T-treatment birds did not sing. During the baseline behavioral recording and during recovery from stereotaxic surgery birds only made calls. After subcutaneous implantation with T-filled implants the birds sang. **B)** The effect of LMAN targeted lesions on song output. Birds that received LMAN targeted chemical lesions sang songs that were simpler and less variable in comparison to sham-lesion controls.

Table 1. Marginal mean values of the song features

Mean (*Std. Error*)	Treatment	Day 21	Day 26	Day 31	Day 36	Day 41
Entropy Variance	Sham Control	0.001 *(0.001)*	0.018 *(0.004)*	0.032 *(0.003)*	0.043 *(0.004)*	0.048 *(0.003)*
	Hit	0.010 *(0.005)*	0.008 *(0.003)*	0.036 *(0.005)*	0.044 *(0.005)*	0.046 *(0.006)*
	Miss	0.008 *(0.006)*	0.043 *(0.013)*	0.067 *(0.005)*	0.084 *(0.017)*	0.074 *(0.018)*
Energy	Sham Control	0.001 *(0.000)*	0.002 *(0.001)*	0.045 *(0.009)*	0.072 *(0.015)*	0.087 *(0.018)*
	Hit	0.001 *(0.001)*	0.006 *(0.003)*	0.025 *(0.005)*	0.058 *(0.010)*	0.079 *(0.013)*
	Miss	0.001 *(0.001)*	0.019 *(0.012)*	0.069 *(0.042)*	0.082 *(0.041)*	0.116 *(0.052)*
Fundamental Frequency Variance	Sham Control	0.079 *(0.035)*	0.151 *(0.032)*	0.218 *(0.033)*	0.170 *(0.025)*	0.177 *(0.022)*
	Hit	0.009 *(0.004)*	0.007 *(0.003)*	0.080 *(0.009)*	0.119 *(0.013)*	0.124 *(0.015)*
	Miss	0.038 *(0.027)*	0.135 *(0.072)*	0.200 *(0.023)*	0.155 *(0.010)*	0.111 *(0.031)*

More specifically, there was no main effect of lesion target on the mean entropy variance ($F(2,9) = 2.064$, $p = 0.183$). However, there was a main effect of treatment day ($F(4,36) = 24.322$, $p < 0.001$, $\eta_{partial}^2 = 0.730$). There was no interaction between treatment day and lesion target category ($F(8,36) = 1.198$, $p = 0.347$). The mean song entropy variance generally increased linearly in response to T-treatment ($F(1,9) = 44.193$, $p < 0.001$, $\eta_{partial}^2 = 0.831$).

Likewise, there was no main effect of lesion target on the mean song energy ($F(2,9) = 0.288$, $p = 0.757$). However, there was a main effect of treatment day ($F(4,36) = 9.740$, $p < 0.01$, $\eta_{partial}^2 = 0.520$). There was no interaction between treatment day and lesion target category ($F(8,36) = 0.176$, $p = 0.915$). The mean song energy (i.e. loudness) generally increased linearly in response to T-treatment ($F(1,9) = 12.519$, $p < 0.01$, $\eta_{partial}^2 = 0.582$).

Finally, there was no main effect of lesion target on the mean fundamental frequency variance ($F(2,9) = 1.166$, $p = 0.355$). However, there was a main effect of treatment day ($F(4,36) = 7.233$, $p < 0.01$, $\eta_{partial}^2 = 0.446$). There was no interaction between treatment day and lesion target category ($F(8,36) = 1.192$, $p = 0.345$). The mean fundamental frequency variance fit a quadratic function in response to T-treatment ($F(1,9) = 28.980$, $p < 0.001$, $\eta_{partial}^2 = 0.763$).

Though the marginal means did not differ by lesion target category the mean coefficient of variation (CV) for each song feature tended to be lower (i.e. less variable) in birds with LMAN targeted lesions (see figure 5.3). More specifically, there was a significant main effect of lesion target on the entropy variance CV ($F(2,9) = 5.831$, $p < 0.05$, $\eta_{partial}^2 = 0.564$). There was also a main effect of treatment day ($F(4,36) = 7.355$, $p <$

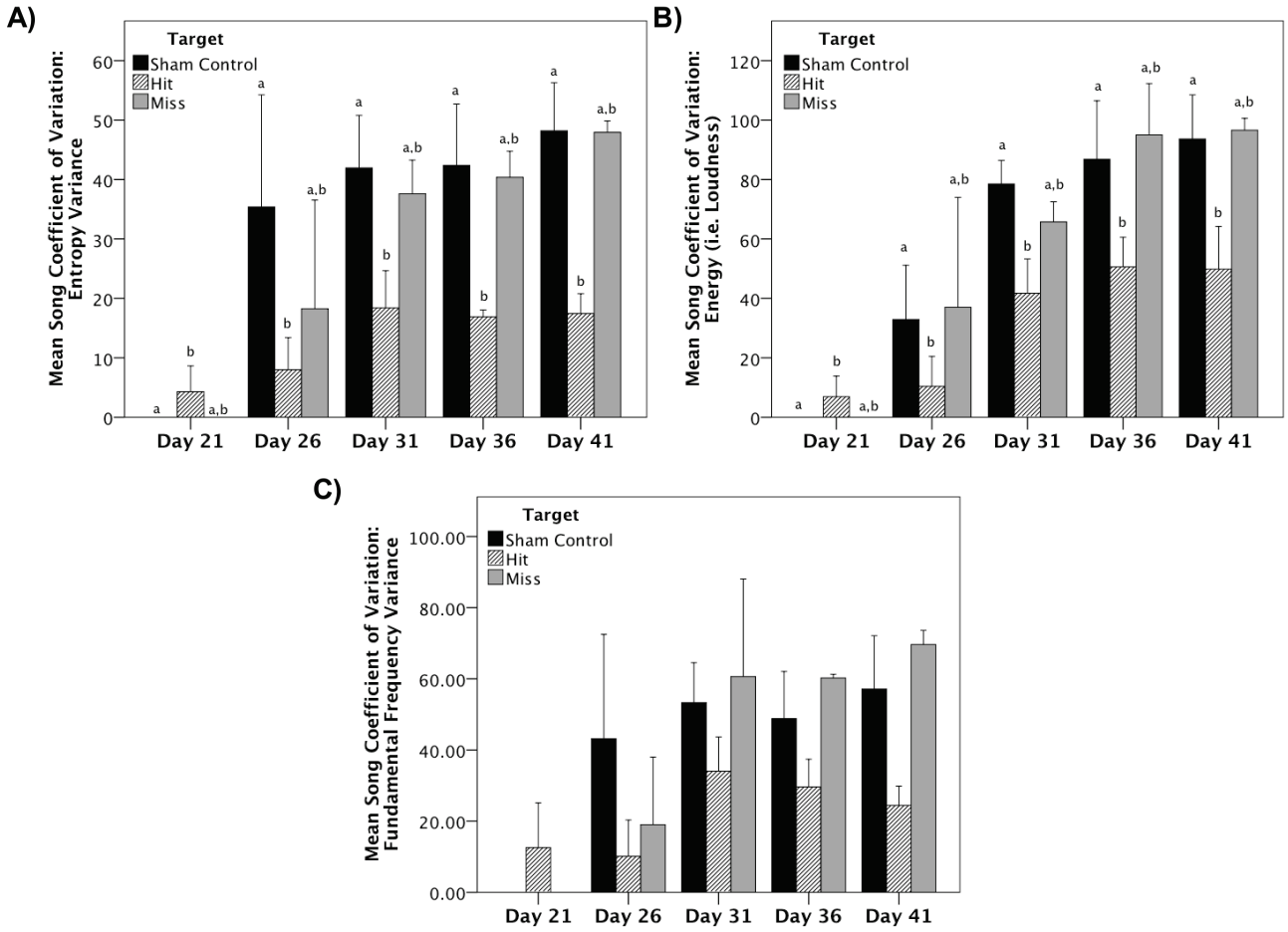


Figure 5.3: The effect of lesion target on song stereotypy. **A)** The effect of treatment on entropy variance coefficient of variation. **B)** The effect of treatment on song energy coefficient of variation. **C)** The effect of treatment on fundamental frequency variance coefficient of variation. Birds with lesion hits sang more stereotyped songs compared to sham treated controls for entropy variance and energy. However, this observation was not significant for fundamental frequency variance.

0.01, $\eta_{partial}^2 = 0.450$). There was no interaction between treatment day and lesion target category ($F(8,36) = 1.037$, $p = 0.409$). The mean song entropy variance CV generally increased linearly in response to T-treatment ($F(1,9) = 22.909$, $p < 0.001$, $\eta_{partial}^2 = 0.718$). Birds with LMAN lesion hits had significantly lower entropy variance CV (i.e. greater stereotypy) in comparison to sham controls ($i\text{-}j_{\text{hit-sham}} = -20.567$, $p < 0.05$); lesion misses did not differ from sham controls ($i\text{-}j_{\text{misses-sham}} = -4.753$, $p = 0.989$) or hits ($i\text{-}j_{\text{misses-hit}} = 15.814$, $p = 0.253$).

Likewise, there was a significant main effect of lesion target on the song energy CV ($F(2,9) = 5.298$, $p < 0.05$, $\eta_{partial}^2 = 0.541$). Furthermore, there was a main effect of treatment day ($F(4,36) = 15.696$, $p < 0.001$, $\eta_{partial}^2 = 0.636$). There was no interaction between treatment day and lesion target category ($F(8,36) = 0.878$, $p = 0.475$). The mean song energy CV increased linearly in response to T-treatment ($F(1,9) = 28.881$, $p < 0.001$, $\eta_{partial}^2 = 0.762$). Birds with LMAN lesion hits had significantly lower energy CV (i.e. greater stereotypy) in comparison to sham controls ($i\text{-}j_{\text{hit-sham}} = -26.473$, $p < 0.05$); lesion misses did not differ from sham controls ($i\text{-}j_{\text{misses-sham}} = -0.490$, $p = 0.989$) or hits ($i\text{-}j_{\text{misses-hit}} = 26.963$, $p = 0.139$).

Surprisingly, there was no main effect of lesion target on the mean fundamental frequency variance CV ($F(2,9) = 2.750$, $p = 0.117$) though lesion hits averaged a lower marginal mean value compared to shams and lesion misses. However, there was a main effect of treatment day ($F(4,36) = 4.833$, $p < 0.05$, $\eta_{partial}^2 = 0.349$). There was no interaction between treatment day and lesion target category ($F(8,36) = 0.780$, $p = 0.523$). The mean fundamental frequency variance CV fit a quadratic function in response to T-treatment ($F(1,9) = 10.497$, $p < 0.01$, $\eta_{partial}^2 = 0.538$).

We tracked the stereotypy of individual syllables in a manner similar to what was done previously in chapter 2. As expected we found a significant main effect of lesion target ($F(1,9) = 4.428$, $p < 0.05$, $\eta_{partial}^2 = 0.496$; see figure 5.4A). Likewise, we found a significant main effect of treatment day ($F(4,36) = 10.372$, $p < 0.001$, $\eta_{partial}^2 = 0.535$). There was no interaction between treatment day and lesion target category ($F(8,36) = 0.993$, $p = 0.440$). Over time, the syllable stereotypy increased in a linear function ($F(1,9) = 18.625$, $p < 0.01$, $\eta_{partial}^2 = 0.674$). Birds with LMAN lesion hits had significantly greater syllable stereotypy in comparison to sham controls ($i\text{-}j_{hit\text{-}sham} = -0.336$, $p < 0.05$); lesion misses did not differ from sham controls ($i\text{-}j_{misses\text{-}sham} = 0.121$, $p = 0.989$) or hits ($i\text{-}j_{misses\text{-}hit} = -0.214$, $p = 0.561$).

For the final day of treatment we inspected each song and tabulated the number of unique syllables per bird. We found a significant main effect of target category on the mean number of unique syllables ($F(2,9) = 8.481$, $p < 0.01$, $\eta_{partial}^2 = 0.653$; see figure 5.4B). More specifically, birds with LMAN lesion hits had significantly fewer unique syllables in comparison to sham controls ($i\text{-}j_{hit\text{-}sham} = -6.800$, $p < 0.05$); lesion misses did not differ statistically from sham controls ($i\text{-}j_{misses\text{-}sham} = 0.200$, $p = 0.999$) or hits ($i\text{-}j_{misses\text{-}hit} = -7.000$, $p = 0.051$).

We also assessed the arrangement, or rather, sequence of song elements by cross-correlating entire sonograms of individual songs on the final day of treatment. We found a significant main effect of target category on the mean song sequence correlation ($F(2,9) = 5.546$, $p < 0.05$, $\eta_{partial}^2 = 0.552$; see figure 5.5). More specifically, birds with LMAN lesion hits sang songs that were more similar in arrangement than sham controls ($i\text{-}j_{hit\text{-}}$

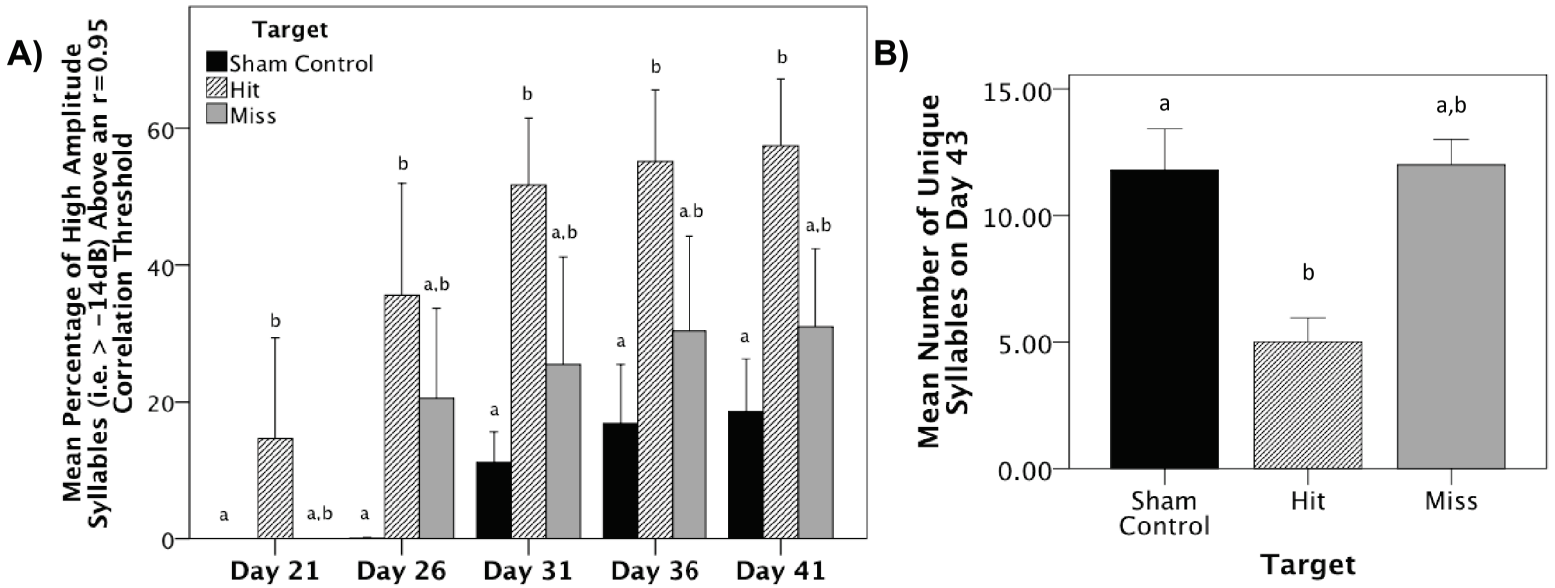


Figure 5.4: Syllable stereotypy and repertoire size. **A)** Birds with LMAN lesion hits had significantly greater syllable stereotypy (i.e. highly correlated) compared to sham controls. **B)** Likewise, it was found that birds with LMAN lesion hits had fewer of unique syllables than sham controls.

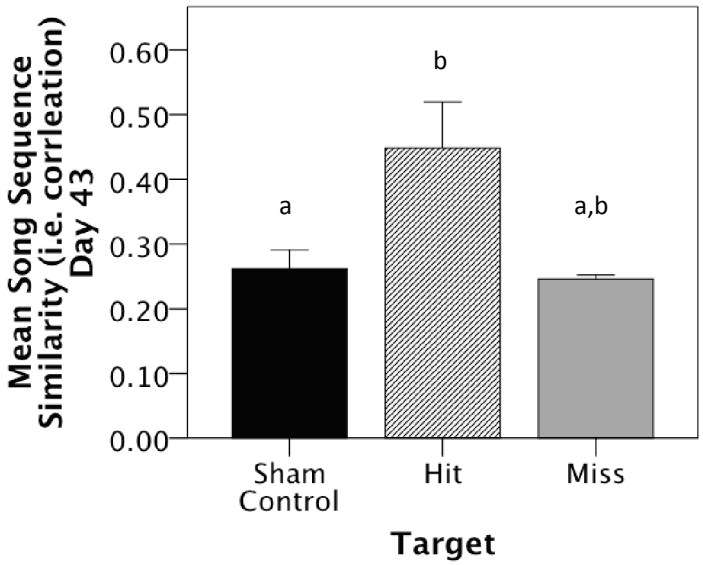


Figure 5.5: The effect of lesion target on the similarity of song arrangements. Birds with LMAN lesion hits sang songs that were more similar to one another compared to sham controls.

$_{sham}$ = 0.225, $p < 0.05$); lesion misses did not differ statistically from sham controls (i-j $_{misses\text{-}sham}$ = -0.016, p = 0.999) or hits (i-j $_{misses\text{-}hit}$ = -0.242, p = 0.111).

Correlations between lesion specificity and song stereotypy/complexity

We wanted to evaluate the effect of lesion size and specificity on the each of the behavioral measures observed. Pearson correlations were run on all birds with anterior forebrain lesions (sham controls were removed). On the final day of treatment (day 43) there were significant correlations between the percent of LMAN lesioned and the song stereotypy and complexity measures (entropy variance CV, syllable energy CV, fundamental frequency variance CV, number of unique syllables, and song sequence similarity; refer to table 2 for results; figure 5.6A-E). Conversely, there were no significant correlations between the lesion volume outside of LMAN and the stereotypy or complexity measures (refer to table 2 for results; figure 5.7A-E).

DISCUSSION:

In this study we evaluated the role of LMAN in T-induced masculinization of adult female canary song. LMAN is a clear target of androgen action based on the high density of androgen receptors in this nucleus (Nottebohm and Arnold, 1976; Arnold and Gorski, 1984; Balthazart, et al, 1992; Bernard, et al, 1999; Fusani, et al, 2000; Gahr, 2000; Ball, et al, 2004). It is also essential for song development in juvenile males based primarily on studies of zebra finches. Sensorimotor learning is supported by input from LMAN to RA not HVC (Olveczky, et al., 2005). To test the role of LMAN we collected baseline song data and confirmed previous findings that adult female canaries do not sing in isolation in the absence of T. We then performed stereotaxic surgeries on all of the

Table 2. Correlations between lesion specificity and size with measures of song stereotypy and complexity

n = 7		Coefficient of Variation Day 43: Entropy Variance	Coefficient of Variation Day 43: Energy	Coefficient of Variation Day 43: Fund. Freq. Variance	Number of Unique Syllables on Day 43	Song Sequence Similarity on Day 43
Percent of LMAN Lesioned	Pearson Correlation	-0.774*	-0.947**	-0.886**	-0.965**	0.853*
	Sig. (2-tailed)	*0.041*	*0.001*	*0.008*	*0.000*	*0.015*
Lesion Outside LMAN Volume (mm^3)	Pearson Correlation	0.482	0.329	0.548	0.200	0.093
	Sig. (2-tailed)	*0.273*	*0.471*	*0.202*	*0.667*	*0.844*

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

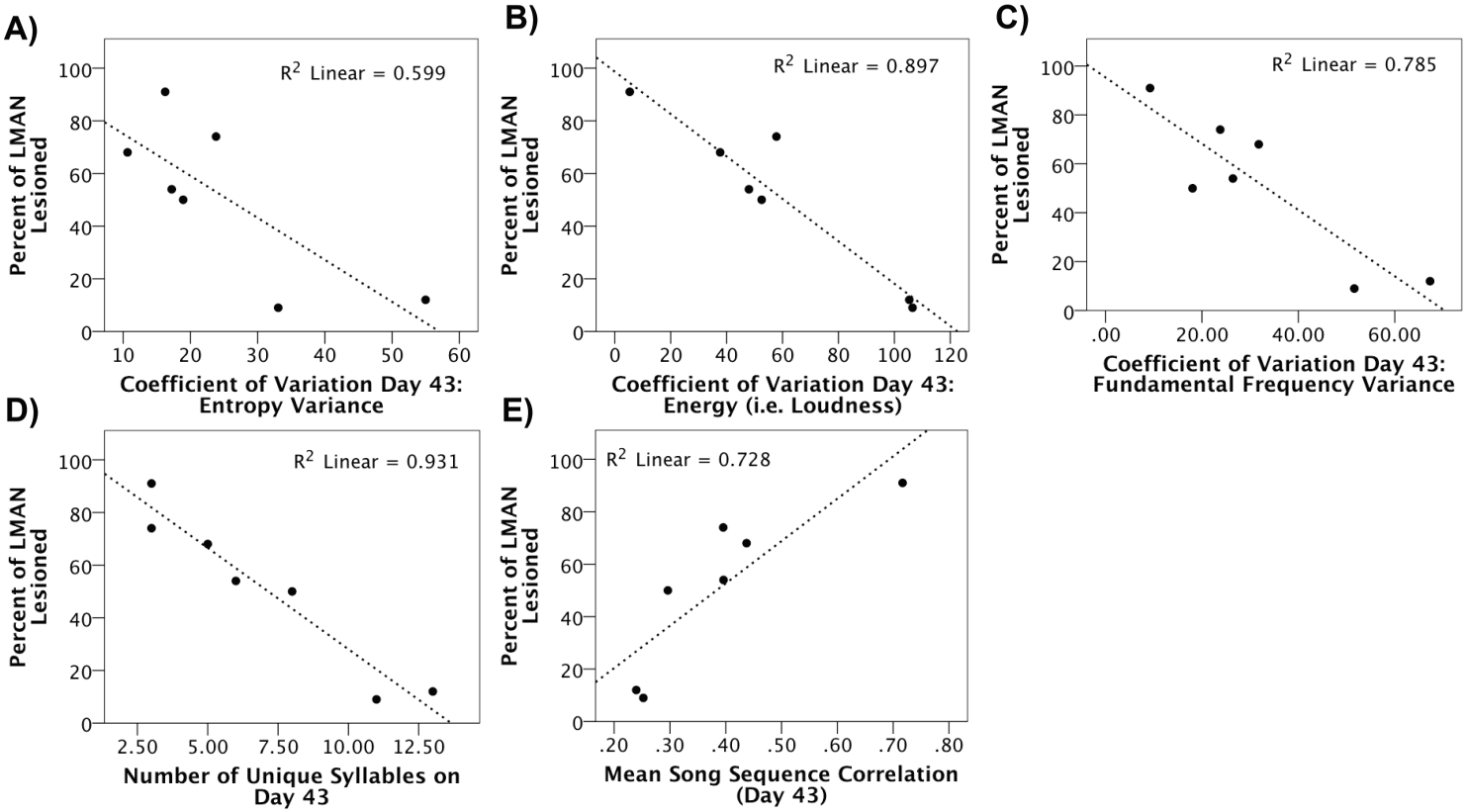


Figure 5.6: The correlation of lesion size and specificity on the stereotypy of song features. It was found that the greater the lesion to LMAN the lower the song feature coefficient of variation, number of unique syllables, and the greater the song sequence similarity (the lower the CV the less variability; **A-E**).

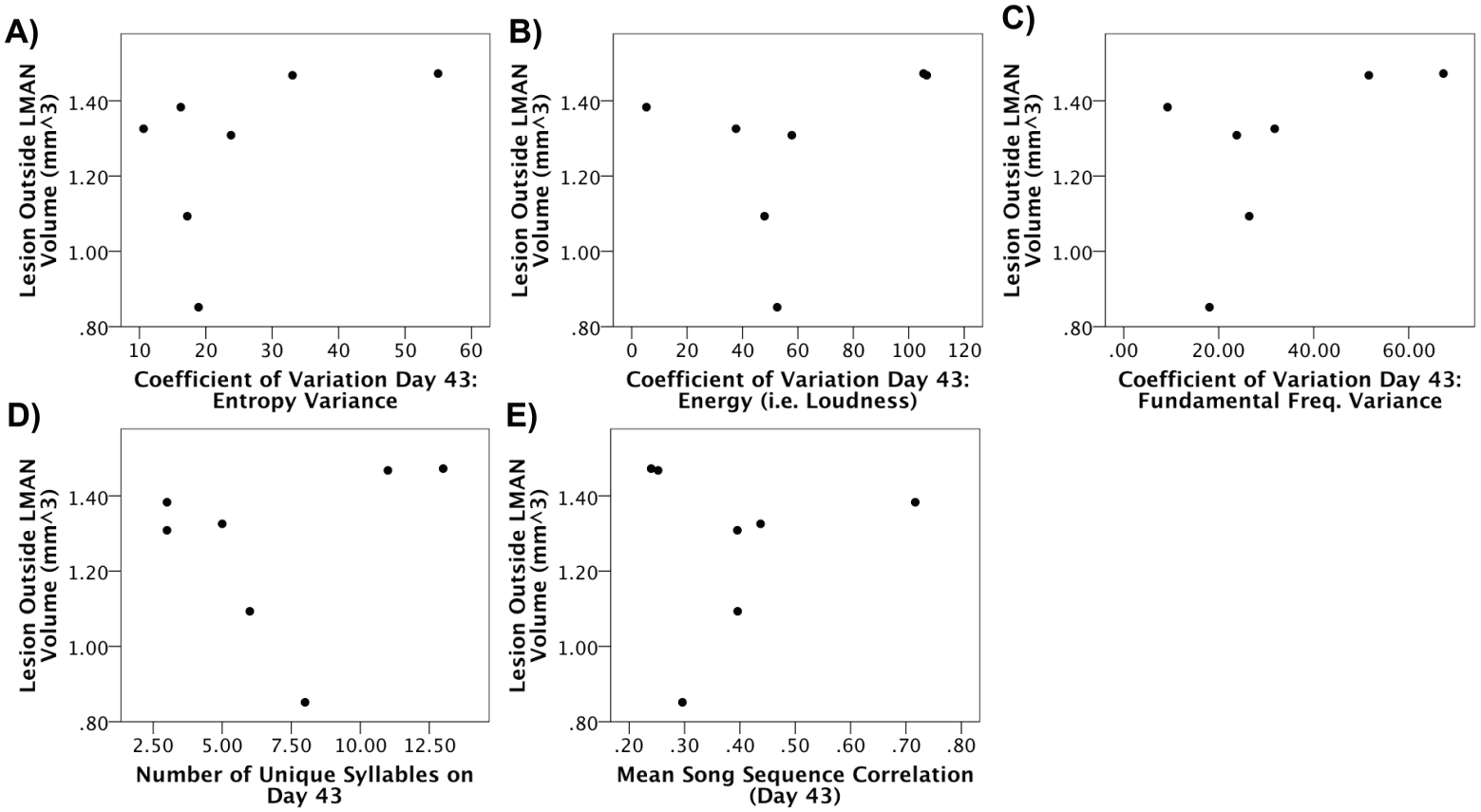


Figure 5.7: There were no significant correlations between the size (i.e. volume) of the lesion outside of LMAN and song features meaning that LMAN alone and not the surrounding nidopallium (or hyperpallium in some cases) is likely to play a role in the stereotyping (i.e. sensorimotor learning) of song elements (**A-E**).

birds; some received chemical lesions targeted toward LMAN and others underwent sham surgeries. Birds were then treated with T after recovery from surgery. T induced significant increases in the rate of song and song output was male-like in syllable repertoire in sham-operated controls. This finding replicates the observation shown in chapter 2 and further confirms that the anterior forebrain (specifically in this case LMAN, the nidopallium, and the hyperpallium) does not control the rate of singing. Recent studies in canaries suggest that T action in the POM plays a key role in promoting increases in song rate and that actions in the forebrain probably regulate aspects of song quality (Alward, et al., 2013).

The masculinization of female canary song is characterized by increases in song complexity and stereotypy over time, however, birds with LMAN lesions tended to sing songs that were simpler, repetitive, and more stereotyped compared to sham operated controls. To evaluate this anecdotal observation of the individual sonograms we measured the acoustic features of song and calculated the coefficient of variation, which gives a broad measure of the level of stereotypy of a given variable. The lower the coefficient of variation the more stereotyped the song feature. Birds with LMAN lesions sang songs with lower coefficients of variation for syllable entropy variance, energy, and fundamental frequency variance in comparison with sham-operated controls. However, this finding was only statistically significant for entropy variance and energy. Conversely though, the raw marginal mean values did not differ between lesion and sham-operated control birds. These findings together suggest that though all the birds an increase in masculinized song in response to T (the mean feature values increase linearly over time which is shown previously in chapter 2 and chapter 4) the way in which these features

vary within and between songs is more stereotyped in birds with LMAN lesions compared to sham-operated controls. This result is consistent with what would be expected if LMAN were functioning in a way that is similar to what has been shown to occur during the initial stages of song development in juvenile male zebra finches.

Furthermore, we found that the individual syllables produced by birds with LMAN lesions during T-treatment were highly similar to one another, more so than sham controls. Additionally, birds with LMAN lesions had a smaller repertoire size compared sham controls. Finally, the syllable arrangement(s) of songs tended to be more similar to one another in LMAN lesion birds compared to shams as noted by higher song sequence correlations compared to sham controls. Male canary song, unlike zebra finch song (the most commonly studied birdsong), is characterized by relatively large syllable repertoires that change across seasons. Likewise, the ways in which these syllables are arranged vary between and sometimes within individual bouts of singing (Markowitz, Ivie, Kligler, and Gardner, 2013). These findings strongly suggest that LMAN is required not just for the generation and development of new syllables (thereby increasing repertoires to male-typical levels) but also for the flexible arrangement of these syllables into multiple song-types. T-induces male-like singing in female canaries after prolonged exposure and is likely acting directly in LMAN to masculinize the syllable and song-type repertoires of T-treated females. This masculinization of syllable repertoires is mostly likely related the variability induced in the individual iterations of syllables. By not being able to alter the acoustic structure of the already limited number of syllables produced (i.e. the increased syllable stereotypy in LMAN lesion animals) new or modified syllables cannot be introduced thereby constraining the ability of T to masculinize song.

In juvenile male songbirds that are learning to sing vocal experimentation is fundamental; it is in this experimentation that song is refined and honed. As previously discussed in chapter 1, plastic song is characterized by high phonologic variability and lacks the stereotypy of adult song (see Marler and Peters, 1982a for detailed studies in sparrows). Young birds engaging in sensorimotor learning to sing, modify as well as drop certain song syllables in order to match the auditory template formed earlier in development during the sensory phase (Marler and Peters 1982a,b; Marler, 1997; Ball and Hulse, 1998; Brainard and Doupe, 2002). Prior to song crystallization if LMAN is inactivated song becomes highly stereotyped and lacks acoustic variability (Olsvecky, Andalman, and Fee, 2005). The aforementioned coefficient of variation results replicates this finding. LMAN lesion in adult female canaries and inactivation in juvenile male zebra finch yield similar behavioral results. The variability that LMAN introduces to vocal output is necessary for song development; in open-ended learners like canaries, this finding has not been clearly confirmed with adult song-learning until now.

Interestingly, it was found that the relative size of the LMAN lesion was significantly negatively correlated with the coefficients of variation for entropy variance, energy, fundamental frequency, and syllable repertoire. Likewise, LMAN lesion size was significantly positively correlated with the mean song sequence correlation. Conversely though, the size of non-specific collateral damage in the nidopallium and hyperpallium was not correlated with any of these measures. These findings tell us that LMAN specifically is the most likely driver of vocal variability in T-induced song learning and masculinization in adult female canaries. By only finding an effect in LMAN specific

lesions T thus appears to be acting directly in LMAN to increase the vocal variability needed to learn and crystallize male-like song in females that do not usually sing.

There are several lines of experimental studies that have analyzed the role of LMAN in song learning; however, most of these studies are limited in scope. Most studies were done in male juvenile zebra finches strictly limiting interpretation to the developmental context of a closed ended learner. However, there are a large number of songbird species where the vocal repertoire changes as the animal ages; with each new breeding season stimulating the addition or deletion of new syllables and the re-composition of familiar songs (e.g. adult canaries and European starlings; Nottebohm, Nottebohm, and Crane, 1986; Eens, 1997). The data presented in this chapter is illustrative in this regard; it suggests that LMANs role does not change in adults versus juveniles and that T in open-ended learners is acting in LMAN (and potentially other areas in the anterior forebrain pathway) to open and/or rather close the sensitive period for song learning and crystallization. Furthermore, T is essential for the actual crystallization of song; in the absence of T, castrate male sparrow song remains highly variable and lacks stereotypy when compared with age yoked T-treated castrates and control birds (Marler, et al. 1988). In adult female canaries T increases song rate, masculinizes the output, and crystallizes song. However, the mechanism(s) by which LMAN and the anterior forebrain pathway *opens* or more likely *closes* T-induced song learning (i.e. masculinization) and crystallization is not well understood, especially for adult song learning. One clear hypothesis based previous observations would be that T increases the motivation to sing by acting directly in the POM (i.e. increased song rate) but acts directly in the song system to reduce variability overtime and crystallize song

(Riters and Ball, 1999;Alward, et al, 2013). In line with this hypothesis we would expect that initially the song that is produced via stimulation of the POM is variable, ostensibly due to LMAN activity in RA. However, over time LMAN to RA inputs are silenced thus driving more stereotyped vocal-motor sequences via HVC to RA inputs. The biochemical changes that T induces in LMAN and the anterior forebrain are multiple, however, there are some candidate mechanisms for how T *closes* song learning. One such mechanism, which will be addressed in chapter 6, is T-induced modulation of excitatory inputs via synaptic modification of modulatory NMDA receptor sub-units.

CHAPTER 6

TESTOSTERONE MODULATES THE EXPRESSION OF N-METHYL-D-ASPARTATE RECEPTOR (NMDAR) SUBTYPES NMDAR-2A AND NMDAR-2B IN THE ANTERIOR FOREBRAIN ADULT FEMALE CANARIES, SERINUS CANARIA

There are a limited number of animal taxa, most notable humans and passerine songbirds, which exhibit vocal motor learning (Jarvis, 2004). Song learning in passerine songbirds occurs in two phases, a sensory phase and sensorimotor phase (Ball and Hulse, 1999). Temperate-zone songbirds are seasonal breeders and seasonal variation in singing is regulated by photoperiod (Schlinger, 1997). Photoperiods drive changes in the singing behavior of male songbirds (Schlinger, 1997). These behavioral changes are related to changing levels of circulating steroid hormones (Ball, et al., 2003). In the absence of photoperiod cues adult male and some female songbirds can respond to exogenous administration of testosterone (T) by singing (Harding, 2003).

In adult female canaries, which respond to T-treatment by singing, T induces growth in the brain regions that control singing and recapitulates sensorimotor learning such that the song becomes more male-like (Nottebohm, 1980; Gahr and Garcia-Segura, 1996). The brain regions that control song learning and production in birds are similar to areas that control vocal-motor production and learning in the human brain (Jarvis, 2004). N-methyl-D-aspartate (NMDA) activity in the anterior forebrain has been demonstrated to be involved in both the sensory phase and sensorimotor phase of song learning in juvenile male zebra finch (Basham, et al., 1996; Wang and Hessler, 2006). NMDA receptors (NMDAR) are activated by the neurotransmitter glutamate, which is an excitatory neurotransmitter present in all vertebrates (Yashiro and Philpot, 2008). NMDA

dependent glutamatergic activity has long been linked as a proximal mechanism of learning and memory (e.g. long-term potentiation/depression, associative learning, etc; White, et al., 1999; Saldanha, Schlinger, Miceyvich, and Horvath, 2004; Yashiro and Philpot, 2008). Furthermore, in male canaries, NMDAR subunit expression is modulated by photoperiod (which is related to increases in circulating levels of T; Singh, et al., 2003). However, what has not been expressly established is if T acts independent of other changes associated with photoperiod to drive NMDAR subtype modulation(s). Furthermore, what also has yet to be established is if this modulation in NMDA mediated synaptic currents is correlated with the recapitulation of T-induced song development in adult female songbirds.

In particular, the likely modulatory subunits that T is up or down regulating are NR2A and NR2B. It has been shown that when NR2B receptors dominate the postsynaptic membrane the overall sensitivity of the cell to synaptic activity is high; thus, even moderate activity is enough to stimulate LTP (Yashiro and Philpot, 2008). However, when NR2A is the dominant modulatory NMDAr the synapse is essentially desensitized to synaptic activity and only strong synaptic currents can induce LTP (Yashiro and Philpot, 2008). It has been shown in slice preparations of the rat hippocampus that the NR2A and NR2B subunits contribute to the determination of the polarity of the synapse (Liu, et al., 2004).

We asked does exogenous T in adult female canaries modulate NMDAR subunit expression (in particular, receptor subtypes NR2A and NR2B) in the song control circuit in correlation with the recapitulation of sensorimotor song learning in adult female canaries? We hypothesize that relative to controls birds treated with T will show an

increase in immunoreactivity for NR2A and a decrease of NR2B immunoreactivity in the anterior forebrain. Furthermore, we expect that the ratio of NR2A/2B expression will increase in the anterior forebrain.

MATERIALS AND METHODS:

Animal Subjects and Experimental Design

Thirty female American singer canaries (*Serinus canaria*) were obtained from a local breeder (Maryland Exotic Birds) and housed in an indoor aviary on an short day 8L:16D (light:dark). Birds were kept in 49 x 95 x 51 cm cages (three to four birds per cage) at Johns Hopkins University, Baltimore, MD and fed canary food and provided water *ad libitum*. After being on this photoperiod for several months female ovaries were regressed and birds were in a photosensitive state. Birds were then transferred to individual sound attenuated chambers (16" x 19" x 20").

Each bird was implanted with a 12mm length Silastic^{TM} (Dow Corning, Midland, MI, USA, no. 602-175; 0.76 mm inner diameter, 1.65 mm outer diameter) tube filled with crystalline testosterone or a blank control. Birds were then placed into individual housing and remained on treatment for 1 week or 5 weeks. Audio and visual behavior was recorded daily for 1 hour at lights on which was 0800hrs. Song rate (the proportion of time spent singing) as well as Weiner entropy variance, a key acoustic feature of song, was measured. Weiner entropy is a measure of the spectral width and uniformity of an acoustic signal and the variance of this measure collapsed across a single bout of singing is indicative of the stereotypy of that song (Tchernichovski, et al, 2001).

Fixation and Histology

At the end of the treatment period (1 week or 5 weeks), birds were euthanized by rapid decapitation and brains were extracted and immersed in fixative solution (5% acrolein). Brains remained in acrolein fixative solution for two hours under agitation. The brains were then washed four times for fifteen minutes each in 1M phosphate buffered saline (PBS) and then transferred to 30% sucrose solution overnight. Once saturated (noted by tissue sinking to the bottom of the sucrose solution), the brains were flash frozen in powdered dry ice for 5 minutes, then placed at -80°C until brain sectioning. Brains were sectioned in the coronal plane with a cryostat at 30μm thickness into four series and we collected every section from the rostral to caudal extent of the brain.

Tissue samples were then processed in random order and the timing of procedure was similar across groups. Brain sections were washed in 0.1 M PBS three times, once in 1% sodium borohydride, then washed three times in 0.1 M PBS, once in 0.5% H2O2 for 1 hour, then the sections were washed three times with 0.1 M PBS. Sections were then incubated for 15 minutes in Avidin blocking solution (Vector laboratories; 1.5ml in 20ml 2% Normal goat serum in 0.3% PBS/T) then washed three times with 0.1 M PBS. Tissue sections were then incubated for 15 minutes in Biotin blocking solution (Vector laboratories; 1.5ml in 20ml 2% PBS/T-NGS) then washed three times with 0.1 M PBS. Sections were then incubated in the primary antibody for NR2A or NR2B (1:2000, AbCam, Cambridge Massachusetts; ab106957 or ab65875) in 20ml 2% Normal goat serum in 0.1% PBS/T at 4ºC for 48 hours. The sections were then washed three times in 0.1% PBS/T, then incubated in biotinylated secondary antibody (Vector laboratories; goat anti rabbit IgG, 1:250 in 20ml 2% Normal goat serum in 0.3% PBS/T) for 1 hour, washed three times in 0.1% PBS/T, incubated in Avidin-Biotin horseradish-peroxidase complex

(Vectastain ABC, Elite; 1:200 in 20ml 2% Normal goat serum in 0.3% PBS/T) for 1 hour, and then washed three times in 0.1% PBS/T. Antibodies were visualized by incubating the sections with the chromagen nickel-enhanced diaminobenzidine (Sigma Fast DAB) for 6-7 minutes. Brains sections were then placed in .01 M PBS solution and then mounted onto gelatin-coated microscope slides. The slides were open-air dried, rehydrated in 0.01M PBS and then serially dehydrated in ethanol at 30%, 50%, 75%, 95%, 95%, 100% for one minute each and a final step in 100% ethanol for five minutes. The slides were then cleared in xylene (Fisher Scientific) and coverslipped with Permount (Fisher Scientific).

Analysis of Immunoreactivity

Area X, the lateral magnocellular nucleus of the anterior nidopallium (LMAN), the medial magnocellular nucleus of anterior nidopallium (MMAN), and HVC (used as its proper name) were identified in coronal sections with reference to Stokes et al. (1974). Images were acquired at 2.5x magnification using a bright-field light microscope (Zeiss Axioskop, Carl Zeiss, Thornwood NY). All images were between 900 KB and 1MB. The light level on the microscope was set exactly the same for each image; exposure time was automated for standard imaging. NR2A or NR2B (NR2A/B) immunoreactivity (ir) were quantified for each region of interest (ROI) in NIH Image J (National Institutes of Health) in a manner similar to what was previously described by Appletants and colleagues (2003). Boundaries of the ROIs were distinct in the pattern of NR2A/B-ir (see figure 6.1). Images were transformed images from RBG color to 16-bit gray scale in NIH image J. Boundary outlines for single ROIs were drawn and surrounding tissue was subtracted

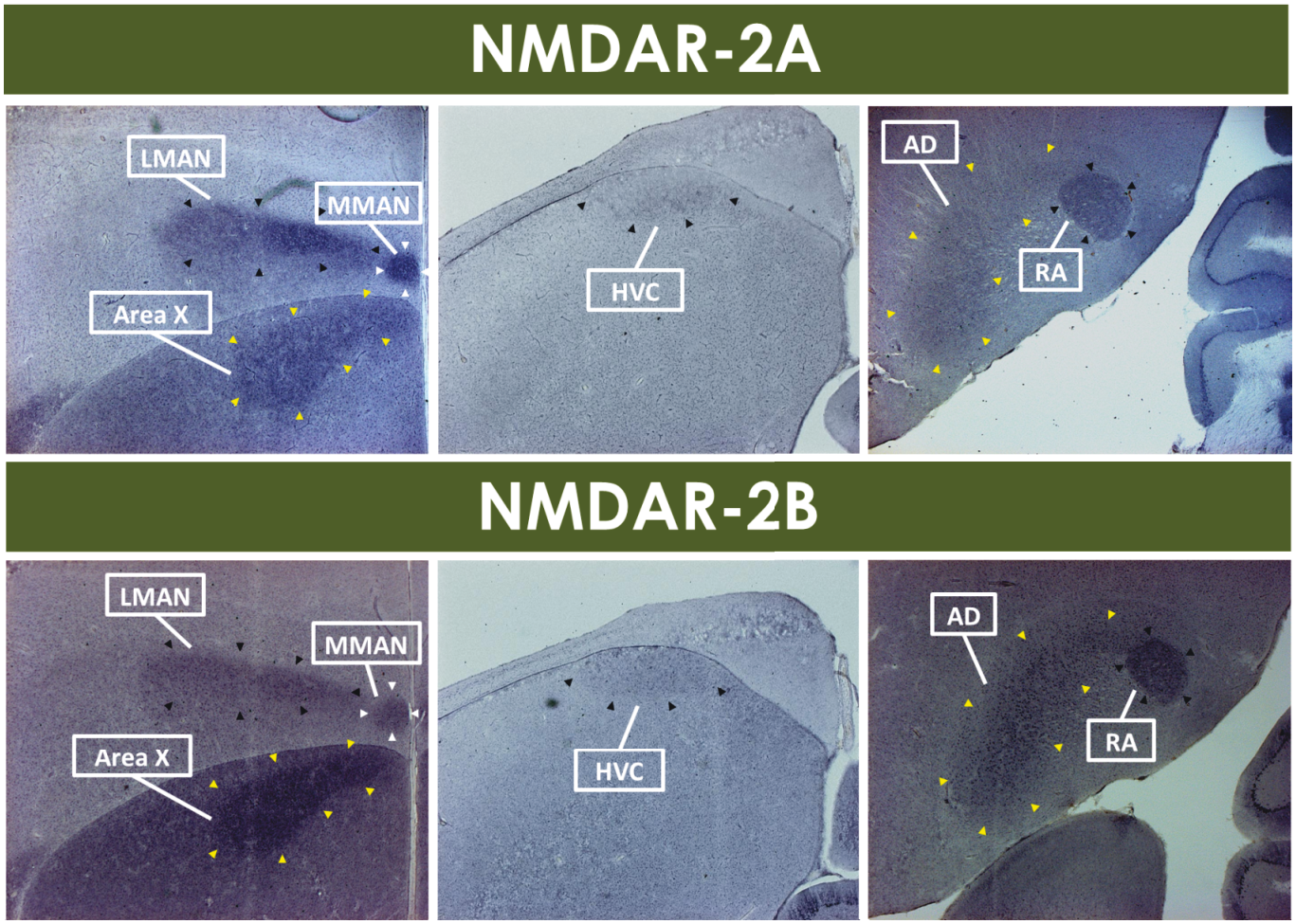


Figure 6.1: NR2A and NR2B immunoreactivity defines the boundaries of key song control nuclei in a manner similar to other criteria such as Nissl stains and NeuN immunohistochemistry. **Abbreviations:** dorsal arcopallium (Ad), lateral magnocellular nucleus of the anterior nidopallium (LMAN), medial magnocellular nucleus of the anterior nidopallium (MMAN), robust nucleus of the arcopallium (RA).

from the image. After the binary images for all sections containing the ROI were obtained, the manual threshold function was used to delineate areas immunoreactive for NR2A/B from background staining. Threshold values were set manually for every individual image to optimize the agreement between blind observations and automated values derived from the automatic threshold feature. The area of the ROI was then calculated automatically (expressed in pixels) as well as the percentage of the total surface area covered by NR2A/B-ir; this measure was called the fractional area covered by NR2A/B-ir immunoreactivity. In addition, we calculated for each subject the relative ratio between the fractional areas covered by NR2A-ir to NR2B-ir in each ROI ($R_{A/B}$ = Fractional Area of NR2A-ir / Fractional Area of NR2B-ir).

Statistical Analysis

To statistically test the histology results we used a 2-way multivariate analysis of variance (MANOVA) with week of tissue collection and steroid treatment (i.e. T-treated or blank control) as the independent variables. Relationships (i.e. correlations) between histology and behavior were tested with linear regression analyses. All statistically significant results were evaluated with respect to $\alpha < 0.05$.

RESULTS:

T-induced modulation of NR2A-ir

As expected we found a significant main effect of treatment in the amount of immunoreactivity measured in the anterior forebrain. In particular, we found that there was significantly more NR2A measured in MMAN ($F(1,29) = 4.697$, $p = 0.040$) and LMAN ($F(1,29) = 5.095$, $p = 0.033$) in T-treated birds compared to controls (see figure

6.2). However, for Area X ($F(1,29) = 0.178$, $p = 0.677$) and HVC ($F(1,29) = 0.017$, $p = 0.898$) there were no significant differences in the amount of NR2A immunoreactivity measured. Similarly, there was no significant main effect of week for Area X ($F(1,29) = 3.541$, $p = 0.072$), LMAN ($F(1,29) = 0.574$, $p = 0.456$), MMAN ($F(1,29) = 1.043$, $p = 0.317$), or HVC ($F(1,29) = 1.555$, $p = 0.224$) in the amount of NR2A immunoreactivity measured. Interestingly there was a significant interaction effect of treatment and week for MMAN NR2A immunoreactivity ($F(1,29) = 11.467$, $p = 0.002$). Post-hoc analysis revealed that during week 1 of treatment there was no effect of treatment on NR2A immunoreactivity in MMAN ($F(1,29) = 1.073$, $p = 0.319$). However, during week 5 of treatment there was a significant effect of treatment on the amount of NR2A immunoreactivity measured in MMAN ($F(1,29) = 11.277$, $p = 0.006$).

T-induced modulation of NR2B-ir

Furthermore, we found that there was significantly more NR2B measured in MMAN in T-treated birds compared to controls (see figure 6.3; $F(1,29) = 6.939$, $p = 0.014$). In Area X we found significantly less NR2B immunoreactivity in T-treated birds relative to controls (see figure 3; $F(1,29) = 13.153$, $p < 0.001$). However, for LMAN ($F(1,29) = 0.194$, $p = 0.664$) and HVC ($F(1,29) = 0.471$, $p = 0.499$) there were no significant differences in the amount of NR2B immunoreactivity measured. Similarly, there was no significant main effect of week for Area X ($F(1,29) = 0.208$, $p = 0.652$), LMAN ($F(1,29) = 1.320$, $p = 0.261$), or HVC ($F(1,29) = 0.907$, $p = 0.350$) in the amount of NR2B immunoreactivity measured. However, there was a significant main effect of week for MMAN NR2B immunoreactivity (there was more total NR2B measured in

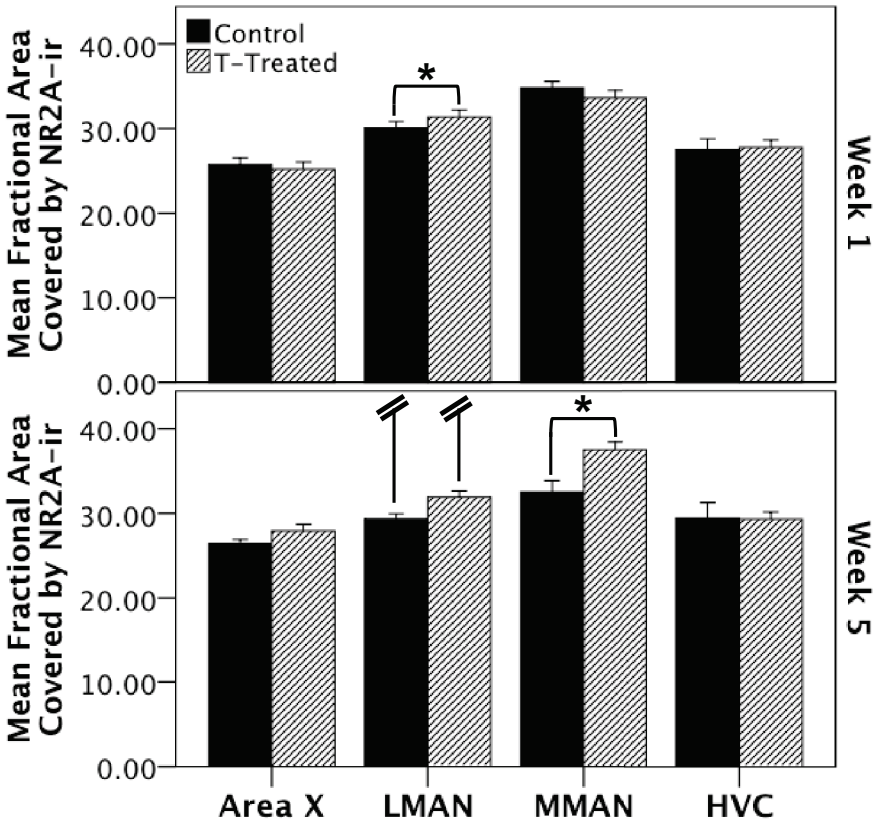


Figure 6.2. The effect of T-treatment on NR2A immunoreactivity (ir) in the anterior forebrain pathway. In LMAN in response to both 1 and 5 weeks of T-treatment there was a significant increase in NR2A-ir relative to blank treated controls. Furthermore, in MMAN after 5-weeks of T-treatment there was a significant increase in NR2A-ir relative to blank treated controls.

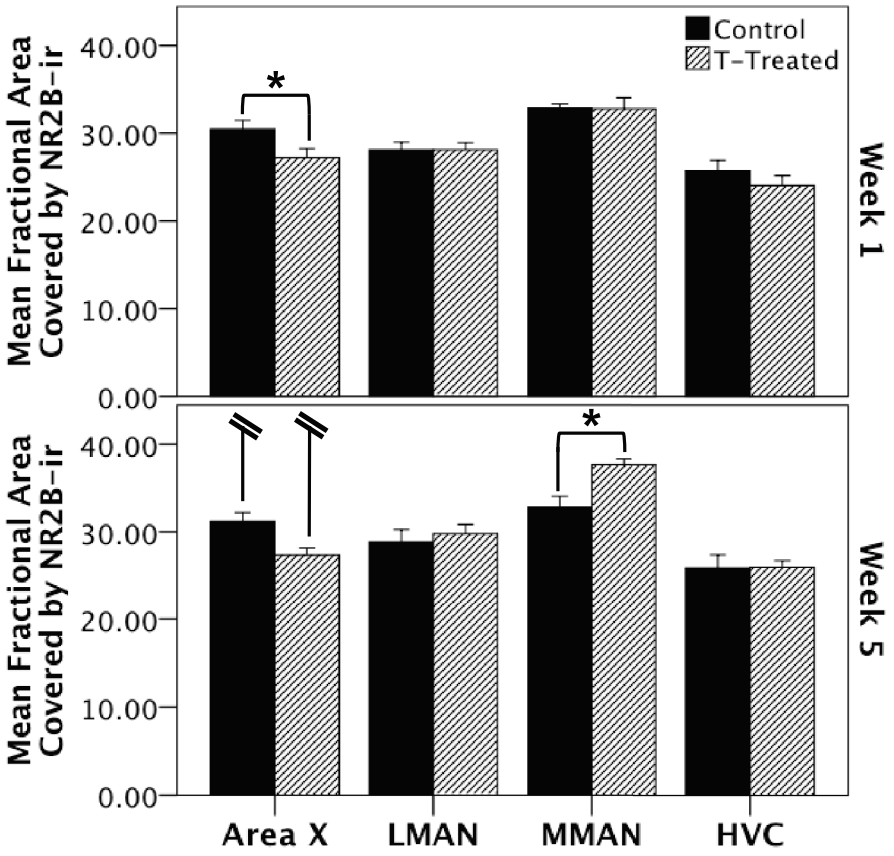


Figure 6.3. The effect of T-treatment on NR2B immunoreactivity (ir) in the anterior forebrain pathway. In Area X in response to both 1 and 5 weeks of T-treatment there was a significant decrease in NR2B-ir relative to blank treated controls. Furthermore, in MMAN after 5-weeks of T-treatment there was a significant increase in NR2B-ir relative to blank treated controls.

week 5 compared to week 1; $F(1,29) = 7.012$, $p = 0.014$). Interestingly there was a significant interaction effect of treatment and week for MMAN NR2B immunoreactivity ($F(1,29) = 7.432$, $p = 0.012$). Post-hoc analysis revealed that for control birds there was no effect of week on NR2B immunoreactivity in MMAN ($F(1,29) = 0.004$, $p = 0.952$). However, for T-treated birds there was a significant effect of week on the amount of NR2B immunoreactivity measured in MMAN ($F(1,29) = 13.404$, $p = 0.003$).

T-induced modulation of the ratio of NR2A to NR2B-ir

A similar pattern was found in the mean ratio of NR2A to NR2B immunoreactivity. We found significant main effect of treatment in the ratio of NR2A to NR2B immunoreactivity in the Area X (see figure 6.4; $F(1,29) = 8.890$, $p = 0.006$). T-treated birds had a greater ratio of NR2A to NR2B compared to controls. However, for MMAN ($F(1,29) = 0.058$, $p = 0.811$), LMAN ($F(1,29) = 0.470$, $p = 0.499$), and HVC ($F(1,29) = 0.184$, $p = 0.672$) there were no significant differences in the mean ratio of NR2A to NR2B immunoreactivity between T-treated and control birds. Likewise, there was no significant main effect of week for any nucleus in the mean ratio of NR2A to NR2B immunoreactivity (Area X: $F(1,29) = 1.322$, $p = 0.261$; LMAN: $F(1,29) = 1.853$, $p = 0.186$; MMAN: $F(1,29) = 1.181$, $p = 0.288$; or HVC: $F(1,29) = 0.010$, $p = 0.920$). Furthermore, there was a significant interaction effect of treatment and week for any nucleus in the mean ratio of NR2A to NR2B immunoreactivity (Area X: $F(1,29) = 1.057$, $p = 0.314$; LMAN: $F(1,29) = 0.273$, $p = 0.606$; MMAN: $F(1,29) = 0.224$, $p = 0.640$; or HVC: $F(1,29) = 1.150$, $p = 0.294$).

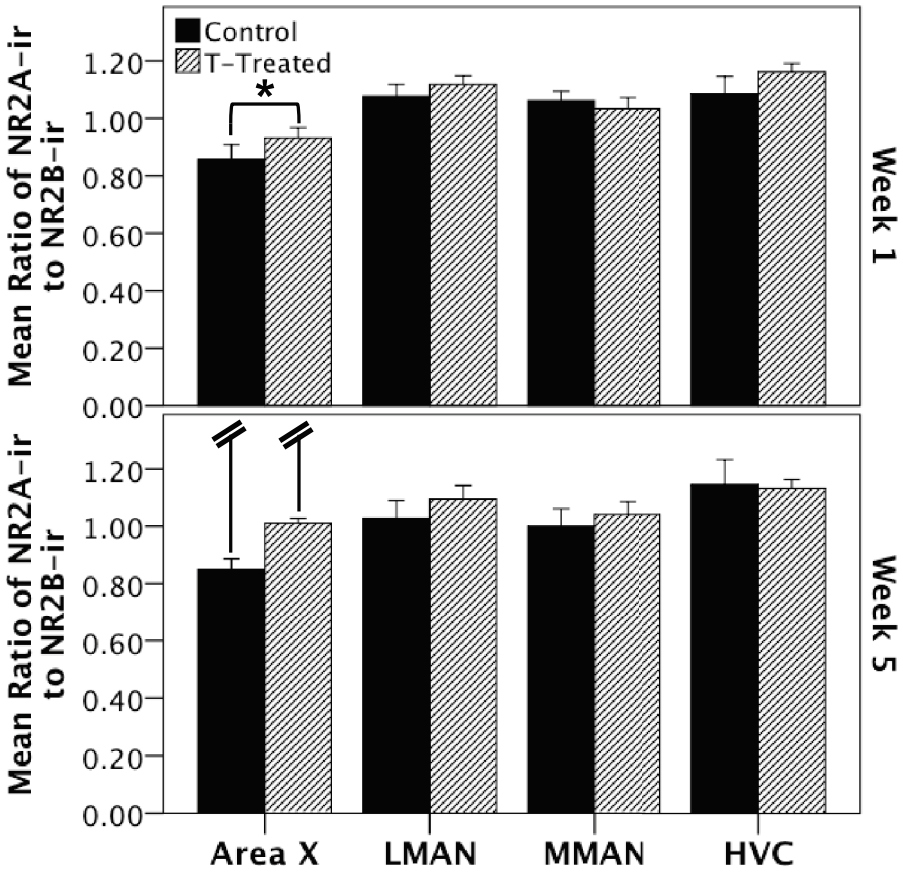


Figure 6.4. The effect of T-treatment on the relative ratio of NR2A to NR2B immunoreactivity (ir) in the anterior forebrain pathway. In Area X in response to both 1 and 5 weeks of T-treatment the relative ratio of NR2A to NR2B-ir was greater in T-treated birds.

Correlation of singing activity and NMDAR immunoreactivity in MMAN

The following data represents only a portion of the data (N = 15; 7 T-treated, 8 controls). As has been previously shown, there was a significant main effect of T on the rate of singing; T-treated females sang more than controls (see figure 6.5A; $F(1,14) = 6.310$, $p = 0.029$). There was no effect or week ($F(1,14) = 1.473$, $p = 0.250$) or an interaction of week and treatment observed ($F(1,14) = 1.010$, $p = 0.336$). Interestingly, linear regression analyses showed that there was a significant predictive relationship between the amount of MMAN NR2B immunoreactivity and the mean proportion of time spent singing (see figure 6.5B; $r^2 = 0.641$, $\beta_{standardized} = 0.801$, $F(1,12) = 21.431$, $p < 0.001$).

DISCUSSION:

In this study we examined the effects of exogenous T in adult female canaries on the pattern of immunoreactivity in NMDAR subunits (NR2A and NR2B) in the song control system in correlation with the induction of song behavior. We hypothesized that relative to controls birds treated with T would show an increase in immunoreactivity for NR2A and a decrease of NR2B immunoreactivity in the anterior forebrain. In addition, we expected that the ratio of NR2A/2B expression would increase in the anterior forebrain.

We found that in adult female canaries T modulates the amount of NMDAr subunit immunoreactivity (ir) in three anterior forebrain nuclei (Area X, MMAN, and LMAN) and in one nucleus (Area X) a T-induced change in the relative receptor ratio. This modulation in expression could be related to T-induced masculinization of song in

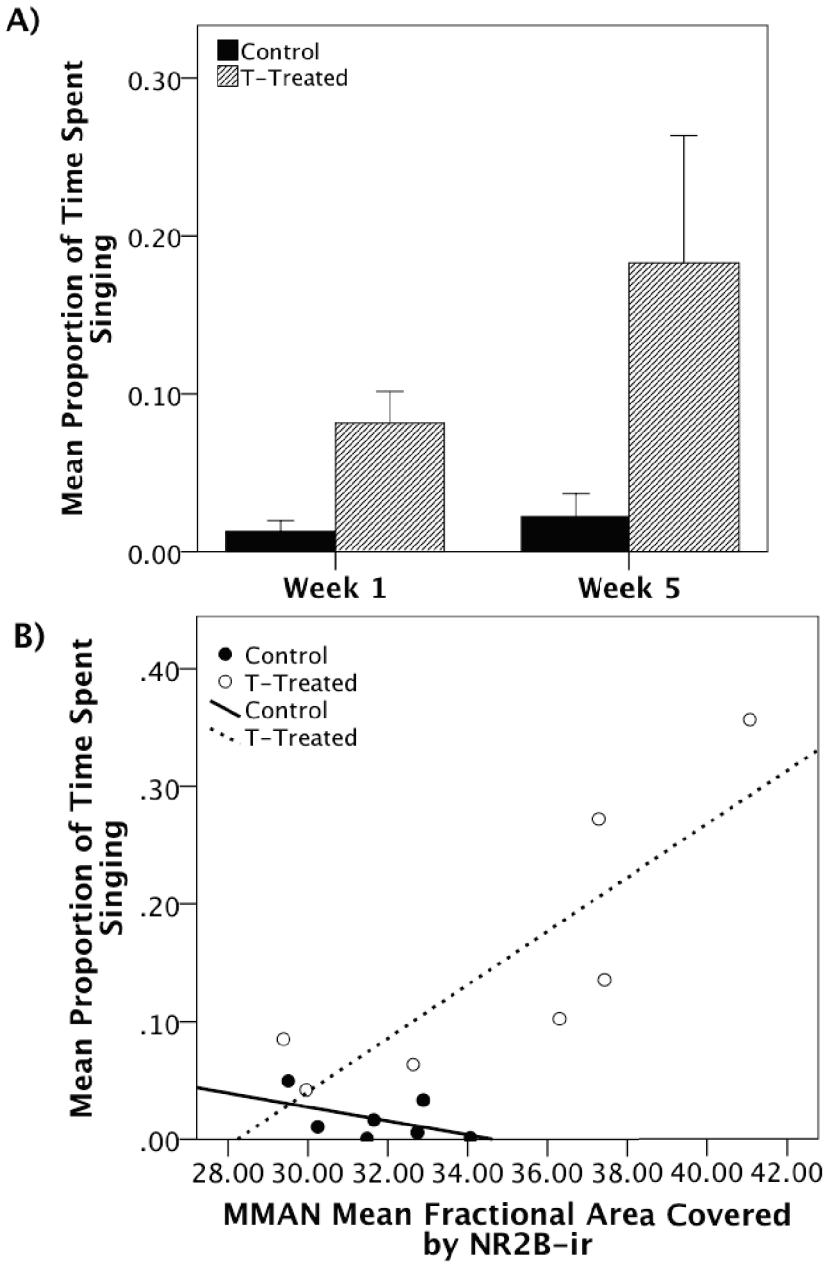


Figure 6.5. The effect of T-treatment on song rate (**A**) and the correlation of song rate and MMAN NR2B immunoreactivity (ir; **B**). These data represent a sampling of the current data.

adult female canaries. By modulating NMDA receptor subunits, brain areas critical for song learning may be sensitized or desensitized (via modulation of synaptic currents). In this regard, the findings in Area X of an increase in NR2A-ir are a challenging finding to interpret.

Early in its discovery the function of Area X was not known because lesions in adulthood did not appear to have a behavioral consequence (Nottebohm, Stokes, and Leonard, 1976). It was not until much later that it was discovered that Area X played a role in the development of song in juvenile males (Scharff and Nottebohm, 1991). Lesions to Area X early in development lead to songs that aberrant in duration and markedly increased syllable variability (Scharff and Nottebohm, 1991). Area X projects indirectly to LMAN via the medial portion of the dorsolateral thalamus (DLM) and LMAN in turn sends a set of projections back to Area X. Area X is a striato-pallidal complex that expresses a wide array of neuron type.

Glutamatergic (NMDA and AMPA) receptors mediate inputs from HVC to Area X spiny neurons (Bottjer, Halsema, Brown, and Miesner, 1989; Farries and Perkel, 2002). Some of these excitatory afferents to Area X spiny neurons have contacts with neighboring pallidal neurons. Pallidal neurons in adult male zebra finch Area X have been shown to inhibit DLM neurons and when that inhibition is removed DLM can activate LMAN via a post-inhibitory 're-bound' (Luo and Perkel, 1999a,b; Leblois, Bodor, Person, and Perkel, 2009). It is possible that by decreasing the relative portion of post-synaptic membranes that show NR2B-ir (consequently increasing the overall ratio of NR2A) in Area X it is possible that DLM is disinhibited thus activating LMAN and turning 'on' sensorimotor song learning. The excitatory afferents from HVC would not

be able to influence downstream inhibition of DLM via Area X pallidal neurons. Thus synaptic modifications that raise the threshold necessary to propagate a signal (in this studies case increases in NR2A receptor ratios via decreases in NR2B-ir) may induce post-inhibitory re-bounds in DLM activating LMAN and turning song learning 'on'.

However, it was also shown in this study that NR2A-ir in LMAN was increased in response to T-treatment. In the previous chapter, chapter 5, it was shown that LMAN is necessary for the acoustic variability and masculinization that is characteristic of T-induced song in adult female canaries. However, what is not clear is what the mechanism turns LMAN and the learning program 'off' (i.e. song crystallization) in response to T. We previously suggested that synaptic threshold modification in Area X may turn LMAN 'on' via dis-inhibition of DLM; however, it is possible that the activation of learning is turned 'off' (i.e. song is crystallized) by increases in LMAN NR2A-ir. The synaptic current threshold may be modified such that it becomes harder for DLM to activate LMAN overtime and what initially may be a highly active nucleus could be slowed to a less active nucleus because the synaptic current strength does not continue to increase over the course T treatment. This would thus reduce variability and increase the stereotypy after the initial increase in vocal variability. However, we cannot test these hypotheses with the current data set so we cannot say definitively the functional meaning of the patterns observed.

Though there are questions that remain about the causal relations between steroid concentrations and particular molecular change, the current study also demonstrates a motor associated pattern of NMDAr immunoreactivity in response to T-treatment. A subset of the data shows that increases in MMAN NR2B immunoreactivity occur in

correlation with increases in the rate of singing. MMAN sends an efference copy of left/right pre-motor RA signals back to HVC. The correlation data suggests that the long-term (5-week) NMDAR-ir modulation we see in MMAN in response to T may be activity dependent as the more birds sang the greater the immunoreactivity in this area we saw.

This current study opens the door to a new avenue of research in the songbird literature and demonstrates a candidate mechanism by which steroids (in this case via NMDA receptor modulation) activate and inactivate adult song learning.

CHAPTER 7

GENERAL DISCUSSION AND CONLUSIONS

Samuel Leonard (1939) and Hugh Hurst Shoemaker (1939) were the first to study the effects of T on a female songbird. It is rumored that Leonards' interest in the effects of T on females was inspired by reports that breeders were selling T-treated females under the premise that they were males since they sang at high rates. It is not clear what spurred Shoemaker's interest in the question as the close temporal proximity of their separate publications (1 month) suggests they were working on the question at the same time, however, at opposite ends of the country; Leonard in the east at Cornell and Shoemaker in the west at Stanford. Unfortunately, however, Leonard and Shoemaker did not have the knowledge of song control system and the process of song learning and its neural control.

The brain areas that control singing in songbirds is well-mapped and consists of a clearly delineated network of specialized forebrain nuclei that shows one of the most pronounced sex differences in brain anatomy in vertebrates. Birds and songbirds have also had a crucial role in revealing that the classic model of sexual differentiation, though attractive in its simplicity, fails to account for sex specific activity of brain cells and sex-specific hormone production and receptivity of neurons independent of plasma steroid levels (Agate, et al., 2003; Maekawa, et al. 2013; Gahr, 2003). The increasing evidence for multiple sex-specific signals and pathways acting in parallel to substantiate differences in male or female phenotypes requires a revision of models of sex differentiation (see figure 1; Arnold, 2004; McCarthy, 2008; McCarthy and Arnold,

2011). This revision is necessary because nature is replete with examples of sex differences in behavior and its neural substrate, many of which have important bearings on patterns we observe in vertebrates in more generally.

Social behaviors like conspecific communication in particular show striking sex-based differences across a wide variety of animal taxa including humans and songbirds. Mate signaling, i.e. singing, in songbirds is an area with particularly well-documented sex differences. A common pattern in mate signaling is male advertisement and female choice. In songbirds, song is sexually dimorphic in many temperate-zone species: males tend to sing more frequently and/or more or more complex songs than females. However, though females do not sing as much (or in some cases not at all) they know a great deal about song and base major life decisions on the information contained therein.

In the differentiation of male and female songbird neural and behavioral phenotypes, hormones play multiple roles particularly in brain development. Fundamental work on brain differentiation and general effects of testosterone on the maintenance of male (song) phenotype in adulthood has been conducted in songbirds (e.g. Arnold, 1975; Wingfield, 1985; Nottebohm, et al, 1987; Marler, et al, 1987, 1988; Nowicki and Ball, 1989; Bernard and Ball, 1996,1997; Soma, et al, 2002; Sartor, et al, 2005). However, we do not fully understand the role(s) of hormones in adult regulation of sexually differentiated song behavior phenotypes, in particular, for female songbirds that must correctly perceive conspecific song.

Our present understanding of song behavior phenotype development and maintenance is biased towards males. We know relatively little about the interplay of hormones, and brain-sex in the maintenance or masculinization (or quite possibly de-

feminization) of the female behavioral phenotype. This gap in our understanding is striking particularly considering that many of the most extreme examples of sexual differentiation involve mating behavior, like song in birds, and is thought to be under the selection pressures of female mate choice and male-male competition for females.

Exogenous administration of T induces a series of changes in the brain and in the periphery, changes that relate to many behaviors including song (Ball et al. 2002). Exogenous administration of T can induce male-typical song in adult female canaries (Leonard, 1939; Shoemaker, 1939). The behavioral effect of T on song behavior in adult female canaries is also associated with particular masculinizing changes in the morphology of the song control system of females (e.g. nuclei volumes, neurochemistry, cytoarchitecture, and vasculature; Nottebohm, 1980; Gahr and Garcia-Segura, 1996; Hartog, et al, 2009). The songbird brain is a dynamic system of recurrent networks and is highly sensitive to the actions of steroids like T. Steroids act in multiple areas in the songbird brain and modulate not only the neural morphology (ostensibly via neuronal birth, migration, and survival) but also the receptor profile of neurons (i.e. synaptic plasticity) in support of song behavior modulation. Furthermore, though the behavioral output of female songbirds can dramatically change in response to T, there are potentially inherent features of song (organized early in development) that make female song perceptually distinguishable from male song. The overarching theme in this text has been that in treating adult female songbirds of particular species with T one can study the effects of testosterone on sensorimotor learning and ipso facto the masculinization (or de-feminization) of song in birds. The data reviewed in this thesis asked three fundamental questions using T-treated female canaries and European starlings as model species:

1) What is the trajectory of vocal development (i.e. sensorimotor learning) in T-induced adult female song and is it different from what occurs in T treated adult male song?
2) Does reproductive state limit the effectiveness of T to induce singing behavior in adult female songbirds?
3) What neural substrates are essential for the induction of song and the recapitulation of sensorimotor learning in adult females?

The simplified version of the answers provided by this text to those three questions is that over time, T-treated male and female canaries exhibited similar patterns of syllable development within and between bouts of singing. Female song was masculinized in duration, stereotypy, and signal amplitude. However, estradiol treated reproductively active female canaries were only mildly sexually responsive to T-treated female song; they responded with greater sexual receptivity to T-treated male song. This is important because it lets us know that female song perception is highly tuned to specific features. Though T masculinized the song output it is possible that there might be some features of male-typical song that females are not able to produce and non-T treated females are able to distinguish this difference based on the song alone. This suggests that there may be some degree of signal phenotype that females are hard-wired (i.e. brain-sex organization) to perceive, evaluate, and respond in kind.

Anterior forebrain activity is essential for the partial masculinization (or de-feminization) of female song. By ablating large portions of LMAN it was demonstrated that T induced song learning could be interrupted and the resulting product is simplified though highly stereotyped. These findings suggest that stereotypy alone is not

characteristic of male-like singing but rather that the specific component parts of song behavior (e.g. syllable repertoire and song-type repertoire) must also be taken into account when describing a birds singing ability as male-typical. Furthermore, it was shown that T modulates excitatory (i.e. NMDA modulatory subunit) receptor immunoreactivity in the anterior forebrain (LMAN, MMAN, and Area X specifically) of adult female canaries in correlation with the induction of singing. These finding are important because these particular forebrain nuclei influence sensory processing and the plasticity of the birds' own song during sensorimotor song learning. The masculinization or de-feminization of a fully feminized brain/song behavior phenotype in adulthood may be at least partly due to synaptic modifications. It may well be that excitatory modulation in the anterior forebrain of T-treated adult females in songbird species that are open-ended learners *starts* and *stops* seasonally driven song behavior.

The particular functional contributions of the anterior forebrain to song behavior were long debated and many theories were championed including error correction and template matching (Brainard and Doupe, 2002; Brainard and Doupe, 2013). However, a growing body of evidence clearly demonstrates that the anterior forebrain is essential for providing the behavioral plasticity necessary for learning by injecting variability in early motor sequence production (Brainard and Doupe, 2013). The findings in this text extend this theory and point to an *activational* sequence that could drive sensorimotor learning in adult female canaries and starlings treated with exogenous T.

T acts in multiple sites in the brain simultaneously to facilitate song behavior (Ball, et al, 2003). For example, T acts in the POM influencing the motivation to sing and increases song rate (Riters, et al, 2000; Alward, et al, 2013). T seems to act in key

forebrain nuclei in the song system such as HVC to influence the quality of song including the addition of new syllables, arrangement of syllables in the new repertoire, and stereotypy of the syllables acoustic features (Alward, et al, 2013; Ball and Nowicki, 1990; Brenowitz, 2004; Meitzen, et al, 2007). However, early in process of T-induced singing (e.g., during the senosorimotor phase of song development as birds reach sexual maturity) LMAN injects the variability needed to produce novel syllables and syllable arrangement. The present data suggests that overtime T acts in LMAN and Area X to silence the LMAN to RA projections thereby reducing the variability of song features (the hypothetical mechanism is discussed in detail in chapter 6). All the while, T acts in HVC to drive the temporal characteristics of song, including starting song and timing transitions between syllable phrases/motifs (Hahnloser, et al, 2002). However, the longer the bird is exposed to elevated T concentrations the greater the relative strength of HVC to RA signaling due to reductions in LMAN to RA input. This slow modulation of vocal-motor control increases the stereotypy of song features over time resulting in the crystallization of the changes to song repertoire (i.e. syllable addition or deletions, as well as new arrangement(s) of syllable motifs).

One point that is not made clear, however, with the current data set is if the effects observed in these studies are due to T per se or perhaps are acting via androgenic or estrogenic metabolites (Saldanha, et al, 1999; Saldanha, et al, 2000; Soma, et al, 2003). In the case of T induced sensorimotor learning in males or the recapitulation of song learning it is most likely that T or its androgenic metabolites are acting to increase vocal plasticity and modifications to song/syllable repertoires. LMAN, unlike HVC (in select species), does not appear to express any form of the estrogen receptor (neither ERα nor

ERβ), however, it does robustly express the androgen receptor (Balthazart, et al, 1992; Bernard, et al, 1999). Thus, any T effects acting via LMAN may be androgenic in nature. LMAN acts a variability generator and injects plasticity into the vocal motor program ostensibly to drive sensorimotor learning; these LMAN effects are likely androgenic.

However, we cannot exclude the possibility of some particular estrogenic effects in HVC, for example, because exogenous T can increase the amount of aromatase activity in the brain (Balthazart, et al, 2001; Fusani, et al, 2001, 2003). Though it has been shown previously that males tend to have greater brain aromatase activity and synaptic expression than females, it is not altogether clear if exogenous T effects in other key nuclei such as HVC are more or less likely due to androgenic effects and not estrogenic effects (Schumaker and Balthazart, 1986; Foidart, et al, 1994; Fusani, et al, 2001, 2003; Peterson, et al, 2005). Furthermore, in female canaries it has been shown that exogenous T administration can increase aromatase activity in the forebrain (Fusani, et al, 2001). In addition, it has been shown that blocking aromatase during T-treatment yields less masculinized songs that are longer in duration and have a greater proportion of slow repeated syllable phrases per song (as opposed to male-typical fast repeated syllable phrases in canary song; Fusani, et al, 2003). Conversely though, syllable repertoires were no different between groups and in light of the data presented in chapter 5 suggests that HVC activity in particular was affected by aromatase inhibition leading to a degradation of the temporal sequencing of syllable phrases despite the male-like development of syllable repertoires (Fusani, et al, 2003).

Unlike both LMAN and HVC, Area X is not known to express regularly either estrogen or androgen receptors (though is has been occasionally reported, see for

example Bernard et al., 1999). However, it has been demonstrated in a number of studies that X can respond to steroid treatment via trans-synaptic effects (Nordeen, et al, 1986; Tramontin and Brenowitz, 2000; Meitzen, et al, 2007). Area X is innervated by steroid sensitive nuclei including HVC and LMAN.

That being said, the exact mechanism(s) that drive T-induced song learning in adult female songbirds are likely multiple and the pattern may differ depending on which nucleus is evaluated and when it is assessed (e.g. pre-breeding versus breeding). Seasonal variation in the hormonal milieu and neural morphology of the song-system in temperate zone-songbirds is one of the most dramatic examples of naturally occurring neuroplasticity there is. This variation in morphology is directly related to seasonal fluctuations in circulating steroids like T, which can induce these changes independent of other cues. This text, in accordance with previous literature on males, affirms that fact in females.

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Melvin L. Rouse, Jr.

MOBILE (540) 818-9965 • E-MAIL – MELROU@GMAIL.COM

1511 BOLTON ST., APT 2 • BALTIMORE, MD 21217

EDUCATION

2007–2013	The Johns Hopkins University Doctor of Philosophy Psychological and Brain Sciences
2004-2005	Boston University Master of Arts General Psychology
2000-2004	Virginia Polytechnic Institute & State University Bachelor of Science Psychology

RESEARCH EXPERIENCE

AUG 2007 – MAY 2014 — Graduate Research (Principle Investigator and Thesis Supervisor: Gregory F. Ball)
Behavioral Endocrinology Group, Department of Psychological & Brain Sciences, Johns Hopkins University

- Avian neuroendocrinology – specialization in temperate zone songbirds
  - Steroid induced plasticity in adult female songbird brain and behavior (canaries and European starlings)
  - Sex differences in response to testosterone in song production and its neural substrate in male and female canaries
  - Male reproductive physiology and photoperiodism in zebra finch
  - Stress effects of mate pair separation in male and female zebra finch

Nov 2005 – Aug 2006 — Oak Ridge Institute for Science and Education (ORISE) Postgraduate Research Intern US Army Medical Research Institute of Chemical Defense, Analytical Toxicology Division, Neurobehavioral Toxicology Branch

- Research of basic proximal mechanisms and behavioral responses to repeated low-level exposure to organophosphorous nerve agents
- Surgical preparation of animal subjects for electroencephalographic (EEG) recording and chronic blood sampling
- Behavioral testing of rodents in acoustic startle chambers and open

field tests

NOV 2004 – MAY 2005	Graduate Research (Principle Investigator & Research Supervisor: Kamilla White), Center for Anxiety and Related Disorders, Psychology Department, Boston University ▪ Directed study for research credit toward masters degree ▪ Cardiac nociception in males with anxiety disorders
JUNE 2003 – DEC 2003	Undergraduate Research Intern (Principle Investigator & Research Supervisor: Tom Ollendick) Minority Academic Opportunities Program (MAOP), Virginia Tech ▪ Clinical treatment of phobias in boys and girls

RESEARCH SKILLS & EXPERTISE

- Performance of small animal surgeries- laparotomy, subcutaneous implantation, stereotaxic chemical lesion, and transcardial perfusion
- Making of and implanting subcutaneous implants from silastic tubing capped with silicone packed with the steroid hormones testosterone or 17Beta-estradiol
- Validation and skilled performance of immunohistochemistry in free-floating tissue
- Western blots in fresh frozen tissue and enzyme-linked immunoassay (ELISA) of blood plasma
- Digital photomicrography of neural tissue for quantitative analysis and publication using light microscopy (NIH Image J and Axioscope by Carl Zeiss)
- Experience in qualitative/quantiataive behavioral and bioacoustics analysis (Avisoft SASlab, Sound Analysis Pro, MATLAB, Raven, and Goldwave ™ sound editor)
- Experimental design and analysis in SPSS

TEACHING EXPERIENCE

Teaching Assistant: duties included grading, leading study review sections, occasional lecturing on topics approved by course lecturer, assisting students with course concerns (study aids, tutoring, and course logistics). Teaching assistantship at Johns Hopkins in the following courses:

FALL 2010 & 2009	Behavioral Endocrinology [course no. AS.200.344] 3 credit hours – approx.78 students/semester
SPRING 2010	Origins of Human Sexual Orientation & Variation [course no. AS.290.420] 3 credit hours – 20 students

SPRING 2009	Foundations of the Mind [course no. AS.200.206] 3 credit hours – 64 students
FALL 2008	Introduction to Cognitive Psychology [course no. AS.200.110] 3 credit hours – 100 students
SPRING 2008	Animal Behavior [course no. AS.200.208] 3 credit hours – 130 students

UNDERGRADUATE RESEARCH SUPERVISION

Mentoring, training and direct supervision of the daily laboratory activities of the following students at Johns Hopkins:

SPRING 2011 – SUMMER 2013	Hannah Wienberg-Wolf Recipient of the David S. Olton Award in Behavioral Biology 2012-13 "Effect of stress from mate pair separation on male zebra finch song behavior and hippocampal glucocorticoid receptor expression in male and female zebra finch"
SUMMER 2010 – SPRING 2012	Sean Dangelmajer Recipient of the Provost Undergraduate Research Award 2011-12 "Modulatory effects of testosterone on the excitatory NMDA inputs of the song system in adult female canaries"
SPRING 2011 – FALL 2012	Kimia Ganjaei "Sex differences in the induction singing in T-treated male and female canaries"

HONORS & AWARDS

SEPT 2011-AUG 2012	▪ Trainee on the NICHD Reproductive Biology Training Grant T32 HD007276, Department of Biochemistry and Molecular Biology, School of Public Health, Johns Hopkins University.
SPRING 2004	▪ Deans List, College of Sciences, Virginia Tech
SUMMER 2003	▪ Summer Research Fellow ~ Multicultural Academic Opportunities Program (MAOP), Virginia Tech
2000-2002	▪ Francis-Floyd Scholarship recipient (2000-2002), Virginia Tech
2000	▪ Ventures Scholar, Virginia Tech

PUBLICATIONS

- Stevenson TJ, Peng KY, Alward BA, **Rouse ML**, Ball GF (in revision). Social regulation of the reproductive neuroendocrine system and singing behavior. Prospective Journal: Proceeding of the Royal Society B.
- **Rouse ML**, Stevenson TJ, Fortune ES, Ball GF (in revision). Reproductive state modulates testosterone induced singing in adult female European starlings (Sturnus vulgaris). Prospective Journal: Hormones & Behavior.
- Madison FN*****, **Rouse, ML***, Balthazart J, Ball GF (submitted). Sex Differences in the Effects of Testosterone on Vocal Development and Song System Morphology in Adult Male and Female Canaries. Prospective Journal: General and Comparative Endocrinology. *** Shared first authorship**
- **Rouse ML**, Ball, GF (in prep). Lesions targeted to the anterior forebrain disrupt vocal variability in testosterone induced sensorimotor song learning in adult female canaries, *Serinus canaria*. Prospective Journal: Developmental Neurobiology.
- **Rouse ML**, Dangelmajer S, Ball, GF, (in prep). Testosterone modulates the expression of N-Methyl-D-Aspartate receptor (NMDAR) subtypes NMDAR-2A and NMDAR-2B in the anterior forebrain of adult female canaries, *Serinus canaria*. Prospective Journal: Hormones and Behavior.
- **Rouse ML**, Weinberg-Wolf H, Ball, GF (in prep). Masculinized female song is only mildly effective in eliciting sexual responses from reproductively active adult female canaries (*Serinus canaria*). Prospective Journal: Animal Behavior.
- Madison FN, **Rouse, ML**, Stevenson TJ, Ball GF (in prep). The effect of social and photoperiodic cues on the hypothalamo-pituitary-gonadal axis and song behavior in zebra finches (*Taeniopygia guttata*). Prospective Journal: General & Comparative Endocrinology.

POSTERS

- **Rouse ML**, Weinberg-Wolf H, Kesner A, Madison FN, Ball GF (2013, June). Mate pair separation induces changes in the acoustic features of song in correlation with hippocampal glucocorticoid receptor mRNA expression in male zebra finch. Poster session presented at Society for Behavioral Neuroendocrinology Annual Conference 2013, Atlanta, GA.
- **Rouse ML**, Dangelmajer S, Ball GF (2012, October). Testosterone induced singing in Adult Female Canaries: modulation of N-Methyl-D-Aspartate receptor subunit expression in relation to male-like singing

behavior. Poster session presented at Society for Neuroscience Annual Conference 2012, New Orleans, LA.

- **Rouse ML**, Madison FN, Ball GF (2012, June). Effects of Variation in Photoperiod and Social Pairing on Song Behavior in Male Zebra Finches. Poster session presented at Society for Behavioral Neuroendocrinology Annual Conference 2012, Madison, WI.
- **Rouse ML**, Madison FN, Ball GF (2012, June). Sex Differences in the Effects of Testosterone on Vocal Development and Song System Morphology in Adult Male and Female Canaries. Poster session presented at Organization for the Study of Sex Differences Annual Conference 2012, Baltimore, MD.
- Alward BA, **Rouse ML**, Stevenson TJ, Ball GF (2012, April). Photoperiodic and Social Regulation of Song Rate and Structure in Male Border Canaries (Serinus canaria). Poster session presented at the annual meeting of the Society-for-Integrative-and-Comparative-Biology (SICB) 2012, Charleston, SC.
- **Rouse ML**, Madison FN, Balthazart J, Ball, GF (2011, November). Effects of Testosterone on Vocal Development in Adult Male and Female Canaries: Sex differences in the effects on catecholamine immunoreactivity and measures of song quality. Poster session presented at Society for Neuroscience Annual Conference 2011, Washington, DC.
- **Rouse ML**, Madison FN, Ball GF (2011, June). Testosterone induced singing in female canaries: sexual receptivity in response to T-treated female canary song. Poster session presented at Society for Behavioral Neuroendocrinology Annual Conference 2011, Queretaro, Mexico.
- Madison FN, **Rouse, ML**, Balthazart J, Ball GF (2010, November). Sex differences in the ability of testosterone to induce increases in the volume of the song control nucleus HVC in canaries. Poster session presented at Society for Neuroscience Annual Conference 2010, San Diego, CA.
- Madison FN, **Rouse ML**, Ball GF (2010, June). Sex differences in the ability of testosterone to induce song in canaries. Poster session presented at Society for Behavioral Neuroendocrinology Annual Conference 2010, Toronto, Canada.
- **Rouse ML**, Stevenson TJ, Fortune ES, Ball GF (2008, November). Reproductive State Modulates the Effectives of Testosterone in Inducing Male-like Song in Adult Female Starlings. Poster session presented at Society for Neuroscience Annual Conference 2008, Washington, DC.
- Roberson MR, Clark ML, Penwell JK, Reynolds, MA, **Rouse ML**, Shafer HF (2006, June). Repeated low-level VX exposure in guinea pigs result in behavioral and electroencephalographic changes that are

consistent with an anxiety-like state. Poster session presented at Bioscience Review 2006, Hunt Valley, MD.

ACADEMIC SOCIETIES

- Member of the Society for Neuroscience
- Member of the Society for Behavioral Neuroendocrinology
- Member of the Animal Behavior Society
- Member of the International Society for Neuroethology

CERTIFICATIONS & TRAINING

- Chemical, Biological, Radiological/Nuclear, and Explosive Incidents Course (CBRNE) Basic Awareness Training – Completed April 21, 2006
- United States Army Medical Research Institute of Chemical Defense – Occupational Health, Allergens, and Zoonotic Diseases Workshop – 3hrs Completed November 22, 2005
- United States Army Medical Research Institute of Chemical Defense – Rodent and Small Animal Handling Workshop – 3hrs Completed November 22, 2005

SERVICE & OUTREACH EXPERIENCE

Student Representative (Spring 2008 – Summer 2013) Graduate Steering Committee (GSC) of the Department of Psychological and Brain Sciences at Johns Hopkins University.

- The GSC is designed to facilitate communication between the graduate student body, the department faculty, and the administrative staff
- Elected by cohort each year to serve as representative
- Maintained open lines of communication between students and faculty, providing constructive input on key issues of concern to the graduate student body

Volunteer Community Outreach Instructor (March 2008 – March 2013) Brain Awareness Week – sponsored by the Department of Psychological and Brain Sciences at Johns Hopkins University, Biology Department at Baltimore Polytechnic High School, and the Society for Neuroscience

- Community outreach program designed to engage high school students with an interest in neuroscience

Volunteer Educational Instructor (September 2006 – May 2007) Virginia Department of Correctional Education: James River Correctional Center/ Powhatan Correctional Center

- Taught a course (Dad's INC) designed to address the issues of parenting for incarcerated persons, including parent rights for inmates, child development, communication, caregivers, reunification, and visitation